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Metabolism during the growth and sporulation of Bacillus cereus var mycoides

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METABOLISM DURING THE GROWTH AND SPORULATION OF *BACILLUS CEREUS* VAR. *MYCOIDES*

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INTRODUCTION

Bacterial sporulation may be considered a differentiation process which may be compared, fundamentally at least, to cellular differentiation in higher organisms. The genetically competent vegetative cell cultivated under the proper environmental conditions undergoes an irreversible transition to the sporulated state (Hardwick and Foster, 1952; Foster and Perry, 1954). If the production of a bacterial spore is metabolically controlled, and there is every indication that it is, then the sporulation phenomenon offers an excellent opportunity for studying differentiation on the biochemical level for which a specific morphological alteration of the cell serves as a readily detectable end point.

Sporogenesis is marked by enzymatic changes within the cell (Hanson et al., 1963a; 1963b). Until recently, the biochemical processes involved in the transition from vegetative cell to spore have been largely neglected. Current investigations have been confined primarily to studies of organisms grown in a glucose-yeast extract-salts medium.

The objective of this investigation has been to establish, confirm, and analyze differences in metabolic patterns emerging during growth and sporulation which might suggest some physiological "common denominator" among aerobic, and possibly all, spore formers. Particular emphasis has been placed on the investigation of pathways related to the metabolism of glutamic acid, and to some extent glucose, and an evaluation of the degree of prominence these pathways might play in growth and sporulation of Bacillus cereus var. mycoides.

LITERATURE REVIEW

Although sporulation is considerably influenced by other environmental factors, the degree of spore formation in any given medium is dependent, to a large extent, on the composition of that medium (Tarr, 1932). Furthermore, the nutritive quantity and quality of a medium can determine the expediency of spore formation. Lehman (1890) and Osborne (1890) reported that the number of vegetative cells and spores was proportional to the initial concentration of the medium. Henrici (1924) concluded that the rate of spore formation was determined by the population in relation to the concentration of nutrients. Brefeld (1881) stated that sporulation proceeded when nutrients in the medium were exhausted. Buchner (1890) demonstrated that sporulation of Bacillus anthracis could be prevented by periodic transfer into fresh medium at appropriate time intervals. Migula (1904) and Bayne-Jones and Petrilli (1933) observed that fresh nutrients added to broth cultures of aerobic bacilli did not stop sporulation. Turro (1891) and Migula (1897) concluded that sporulation was not due to the exhaustion of nutrients but to the accumulation of metabolic products.

To understand these seemingly paradoxical observations, consideration must be given to commitment to sporulation. Hardwick and Foster (1952) showed that the addition of glucose to an early log phase culture of aerobic bacilli prevented sporulation. When glucose was added at later stages of growth, its effect was progressively less, and sporulation could not be prevented even if the cells were washed and shaken in distilled water. Powell and Hunter (1953) attributed sporulation in distilled water

to nutrients furnished by massive lysis of the suspended vegetative cells. Perry and Foster (1954) reported that under their experimental conditions lysis was not involved during the sporulation of Bacillus mycoides in distilled water. Most, if not all, current investigators of bacterial sporulation concede that at some stage of cultural growth the genetically competent cells in the proper environment are irreversibly committed to spore formation, the commitment being directly related to the synthesis of enzymes required for the formation of spore material. Commitment, therefore, does not refer to an immediate, visible, morphological alteration of the cell, but to a metabolic diversification which predisposes the organism to spore formation. Thus, cells supplied with fresh nutrients before attaining the commitment stage will continue to vegetate, while those already committed will sporulate.

Before the development of synthetic media for studying sporulation, complex media containing peptones were the principal media investigated for spore yield. Williams (1931) and Brunstetter and Magoon (1932) found that the percentage of spores formed in a peptone medium varied inversely with the amount of peptone, and Williams (1931) concluded that a depletion of nutrients was more important in spore formation than the accumulation of metabolites. Tarr (1932) noted that few spores were formed when large amounts of nutrients were used, while media containing only small amounts of amino acids gave good sporulation. Roberts and Baldwin (1937) observed that charcoal added to a Bacto-peptone medium stimulated sporulation significantly and believed that some commercially prepared peptones contained substances inhibitory to sporulation. By treating peptone media

with activated charcoal, Roberts and Baldwin (1942) removed the antisporulation factors. Foster et al. (1950) obtained little or no growth of Bacillus larvae in peptone and other complex media and attributed this to the content of inhibitory substances in such media and not to an inadequate nutritional content. Treatment of the media with activated charcoal and soluble starch yielded a substantial increase in growth and sporulation. Hardwick et al. (1951) suggested that the antisporulation factors in complex organic media were fatty acids. Foster and Heiligman (1949a) compared the sporulation of B. mycoides in nine different, relatively dilute, complex media and concluded that asporogenic complex media were deficient in minerals for sporulation but sufficient for vegetative growth.

Early attempts to demonstrate sporulation of aerobic bacilli in chemically defined media were unrewarding (Williams 1929). However, further studies with synthetic media led Williams and Harper (1951) to conclude that sporulation in chemically defined media was generally better than in complex media. Roberts (1934) tested some 60 combinations of nutrients for the sporulation of Bacillus subtilis and found the sole satisfactory medium, yielding a 60-70 percent spore crop in 5 days, to contain: $NH_4H_2PO_4$, K_2HPO_4, KH_2PO_4, $MgSO_4$, KCl, asparagine and levulose. Gladstone (1939) grew several strains of B. anthracis in media containing amino acids, salts and glucose and found that growth of most of the strains used was stimulated by glutamine. Brewer et al. (1946) developed a synthetic medium for growth and sporulation of B. anthracis which contained glucose, glutamine, 18 amino acids, nucleic acid components, sodium bicarbonate and thiamine. These investigators found that bicarbonate was

required for growth and emphasized the importance of various metal ions on growth. Fabian and Bryan (1933) showed that univalent cations of chloride salts stimulated the sporulation of aerobic bacilli in liquid medium. Foster and Heiligman (1949b) and Perry and Foster (1955) achieved good sporulation of Bacillus cereus with a glucose glutamate-salts medium (CGS medium). Although good growth was obtained without glucose, only negligible sporulation was observed. Alanine was found to be inhibitory to sporulation even in the presence of glucose.

For several strains of B. cereus, Grelet (1955) developed a synthetic medium of mineral salts, glutamate, alanine, valine, leucine, isoleucine and glucose. By varying the initial concentrations of the five amino acids, Grelet demonstrated that these strains required glutamate and alanine, while valine, leucine and isoleucine stimulated growth. Good sporulation occurred when alanine became limiting. When valine, leucine or isoleucine became limiting, good sporulation was obtained even in the presence of excess alanine. Although no sporulation was obtained with 0.8 percent glutamate as the sole carbon source, lesser quantities (0.4 and 0.2 percent) provided almost total sporulation. Similar results were obtained with carbon-limiting malate, fumarate, succinate and acetate. Grelet (1955) concluded that sporulation was produced when the limiting carbon was consumed.

Dutky (1947) developed several media for growing Bacillus popilliae and Bacillus lentimorbus containing glucose and fructose as carbon sources, however, none was adequate for sporulation. Knight and Proom (1950) characterized the nutritional patterns for many species of Bacillus

among which was Bacillus cereus var. mycoides requiring an organic nitrogen source.

Manganese was discovered to be required for sporulation in concentrations exceeding those for growth (Charney et al., 1951). Stockton and Wyss (1946) demonstrated a manganese-requiring proteinase in B. subtilis. Hardwick and Foster (1952) showed that some vegetative cell enzymes were sacrificed for spore synthesis. Curran and Evans (1954) found that suitable additions of manganese to liquid media greatly increased total spore production in 3 species of Bacillus. The relation between these observations led Curran and Evans to suggest that manganese may aid spore formation "by contributing to the reserve of intracellular enzyme proteins believed . . . to be the basic material for spore synthesis."

The synthetic media currently employed by investigators of bacterial sporulation are generally of glucose-yeast extract-salts sometimes supplemented with one or more amino acids. Probably the most well known of the media used for studying the biochemical changes during the sporulation of Bacillus cereus T is the "G" medium (Hanson et al., 1961).

The advent of simple media which support good growth and sporulation has made the study of the biochemical events during sporogenesis more feasible. Gary and Bard (1952a) compared the fermentative capacity of cells of B. subtilis grown in a simple glucose-yeast extract-salts medium with that of cells grown in a complex tryptone-yeast extract-glucose medium. Cells grown in the complex medium (C-cells) were capable of vigorous respiration and glucose fermentation (glycolysis) but could not oxidize glucose to completion, while cells grown in the simple medium

(S-cells) respired actively, were incapable of glucose fermentation, yet oxidized glucose to completion. Divergent metabolic pathways and possibly, qualitative as well as quantitative enzymic differences were suggested by these findings. C-cells were unaffected by potassium cyanide indicating the absence of a cytochrome system. The 2 types of cells were found to possess quantitative differences in aldolase, glyceraldehyde-phosphate dehydrogenase and cytochrome oxidase (Gary and Bard, 1952b). Gary et al. (1954) concluded that C-cells developed both the pentose and Embden-Meyerhof pathways but possessed an incomplete cytochrome system, while S-cells developed only the pentose shunt and contained an active cytochrome system. The addition of a suitable hydrogen acceptor allowed S-cells to dissimilate glucose via glycolysis. Metabolic pathway selection within the cell apparently can occur, which is dependent to a large extent on the nutritional environment. Such metabolic shifting may occur more often than is realized (Gary et al., 1954). Tinelli (1955a) observed that spores produced in media containing different carbon substrates were quantitatively and qualitatively similar.

Keilin and Hartree (1947) found that spores contained only 6 percent as much cytochrome as corresponding vegetative cells. Spencer and Powell (1952) suggested that a flavoprotein enzyme system in spores replaced the cytochrome respiratory enzymes found in vegetative cells. Doi and Halvorson (1961) confirmed that the main pathway of electron transport in vegetative cells was by a particulate cytochrome system as compared with a soluble flavoprotein oxidase in spores.

Wang and Krackov (1962), utilizing cells of B. subtilis grown in the

same media used by Gary and Bard (1952a, 1952b) and using labeled glucose and gluconate, concluded that this organism degraded glucose aerobically through both the glycolytic and pentose pathways regardless of their cultural history. These findings do not agree with those of Gary and Bard (1952a, 1952b) and Gary et al. (1954). Goldman and Blumenthal (1959, 1960) showed that resting vegetative cells of Bacillus subtilis Marburg used both the Embden-Meyerhof and pentose pathways while vegetative cells of B. cereus T and cells of B. cereus T containing refractile spores revealed no significant utilization of the pentose pathway.

Beck and Lindstrom (1955), using a complex medium which provided rapid, heavy growth of nonsporulating cells, demonstrated an active TCA cycle in intact cells and cellular extracts of B. cereus harvested after 16 hours of incubation. Although whole cells exhibited acetate oxidation, cellular extracts did not. The failure to detect citrate synthesis by cellular extracts suggested the loss of either the acetate activating enzyme or condensing enzyme.

Nakata and Gollakota (1959) and Nakata and Halvorson (1960) correlated changes in pH of a culture medium containing glucose and yeast extract with microscopic observations of stained preparations of B. cereus T. A rapidly decreasing pH resulting during growth, which reached a minimum about the end of the logarithmic phase of growth, was followed by a rapidly increasing pH which reached a level higher than the initial pH of the medium. The increase in pH was accompanied by cytological changes characteristic of cells about to enter the sporulated state. The basal medium containing no glucose revealed no significant changes in pH during

growth. The decrease in pH of the medium with glucose was attributed to the dissimilation of glucose to accumulating organic acid intermediates which were subsequently utilized as sporulation began. Gollakota and Halvorson (1959, 1960, 1963) observed that alpha-picolinic acid and ethyl malonate could suppress sporulation while allowing normal growth to proceed. Nakata and Halvorson (1960) showed that in the absence of sporulation, due to the addition of alpha-picolinate or malonate, the pH of the culture medium decreased and remained at a minimum level even after prolonged incubation. Qualitative tests revealed the only significant organic acids in the filtrate to be pyruvate and acetate, the latter predominating. These observations led to the conclusion that the accumulation of acetic acid during growth was due to the lack of a complete oxidation mechanism in vegetative cells of the test organism, B. cereus T. The rapid utilization of acetate in the medium was concluded to be the result of a new enzyme system, absent or nonfunctional during growth but appearing during sporogenesis.

Acetate was found to be oxidized to carbon dioxide and also converted to poly-beta-hydroxybutyrate during sporulation (Nakata, 1962). Hanson et al. (1963a) observed that fluoroacetate if added during early growth inhibited sporulation but not vegetative growth. The lack of an inhibitory effect on sporulation by fluoroacetate added during sporogenesis indicated the absence of an operative TCA cycle.

Amaha et al. (1956) found that 1 strain of Bacillus coagulans var. thermoacidurans grown in complex media required TCA cycle intermediates to stimulate sporulation and presumed that the citric acid cycle was

involved in sporulation by providing energy for the process. Extracts prepared from cells of B. cereus T harvested from a glucose-yeast extract medium at stages of vegetative growth and sporulation were tested for some of the TCA cycle enzymes (Hanson et al., 1963b). Acetokinase and phosphotransacetylase were found to be present in extracts of both vegetative cells and sporulating cells. The activities of condensing enzyme, aconitase, succinic dehydrogenase, fumarase and malic dehydrogenase were found to be greatly exaggerated in extracts of sporulating cells; extracts of nonsporulating cells displayed little or no activity of these enzymes. These data indicate that TCA cycle enzymes are formed during sporogenesis and are required before sporulation occurs.

Dipicolinic acid (DPA) was discovered to be present in bacterial spores by Powell (1953). The biosynthesis of this compound, peculiar to bacterial spores, has been investigated during the sporulation of at least 2 species of aerobic bacilli (Perry and Foster, 1955; Martin and Foster, 1958; Powell and Strange, 1959). Martin and Foster (1958) suggested that the carbon skeleton of DPA originates from a $C_4 + C_3$ condensation, such as aspartate condensing with pyruvate or alanine condensing with oxalacetate. Alpha-picolinate and fluoroacetate were shown to inhibit aconitase and sporulation in B. cereus T, the inhibitory effects being reversible by citrate, cis-aconitate, isocitrate and succinate but not by alpha ketoglutarate or fumarate. These results led Gollakota and Halvorson (1959), Halvorson (1960) and Hanson et al. (1963a) to believe that succinate, derived from acetate via the glyoxylate cycle, may be the key intermediate in DPA synthesis and spore material.

Hardwick and Foster (1953) compared transaminases, deaminases, and dehydrogenases of certain amino acids and oxidative enzymes of certain organic acids in vegetative and sporulating cells of aerobic bacilli. Since enzymes involved in carbohydrate oxidation appeared to be lost faster than those of amino acid metabolism, it was concluded that the former were sacrificed during sporogenesis providing amino acids for the synthesis of spore substances. Thus, the amino acid enzymes are retained to participate in biosynthetic reactions leading to spore formation. Levinson and Sevag (1954) found strong glutamate-aspartate transaminase activity in extracts of spores and vegetative cells.

Glutamate is known to occupy an important position in the biosynthesis of certain peptides of many species of Bacillus. An extracellular peptide composed only of D-glutamate was found in B. subtilis by Bovarnick (1942). Capsular polypeptides of D-glutamate and L-glutamate have been observed to be produced by B. anthracis (Hanby and Rydon, 1946; Housewright and Thorne, 1950; Thorne et al., 1952) and B. subtilis (Thorne et al., 1954; Housewright and Thorne, 1950). Strange and Powell (1954) showed glutamate to be a peptide constituent only in spore extracts and germinating exudates of Bacillus megatherium, B. subtilis and B. cereus.

Glutamate has also been shown to be involved, one way or another, in metabolic reactions of species of Bacillus. Weinberg (1955) resuspended cells harvested at 10 or 24 hours in phosphate buffers of pH 7.0 and pH 8.0 with and without glutamate. The cells were shaken and observed for sporulation at 12, 24, and 36 hours. In buffer solutions without glutamate only slight sporulation was observed even in the presence of

manganese. Cells sporulated faster in the buffers containing glutamate but always slower in the buffer of pH 7.0 with or without glutamate. In the neutral buffer an alkaline pH shift was noted to occur provided glutamate was present. Krask (1953) demonstrated that methionine sulfoxide, an analogue of glutamate, inhibited sporulation of B. subtilis in a glucose-glutamate-salts medium without affecting growth and proposed that the amidation of glutamate to glutamine was involved in the synthesis of spore material. Keynan et al. (1954) found that glutamate or glutamine markedly stimulated glucose and pyruvate utilization under aerobic conditions and concluded that these compounds enhanced the non-oxidative conversion of pyruvate to acetoin. Acetoin has been related to the synthesis of poly-beta-hydroxybutyrate (Kominek et al., 1963), a lipid synthesized during growth and subsequently utilized during sporulation of Bacillus species (Tinelli, 1955b and 1955c; Stevenson et al., 1962; Nakata, 1962).

Wiame and Pierard (1955) using a glutamate-salts medium revealed that the ability of B. subtilis to utilize ammonium as a sole source of nitrogen corresponded to the presence of glutamate dehydrogenase. A mutant, unable to use ammonium and lacking glutamate dehydrogenase, oxidized glutamate quite actively. Alpha and gamma-aminobutyrate were oxidized only slowly and glutamate decarboxylase was nonexistent. Although not demonstrated, it was suggested that alpha-ketoglutarate served as the carbon source, being formed via glutamate-alanine transaminase.

Young and Fitz-James (1959) found that the free amino acids of B. cereus were primarily glutamate and alanine. Although the alanine

concentration did not change during sporulation, the glutamate concentration decreased during sporogenesis. A sharp increase in glutamate was noted at the beginning of sporogenesis followed by a rapid decrease during spore formation. Millet and Aubert (1960) related the rise and fall of free glutamate with the production of N-succinylglutamate formed at the time of glucose exhaustion and just prior to the appearance of DPA.

Thorne et al. (1952) demonstrated that 3 highly virulent strains of B. anthracis grown on a solid complex medium or a solid synthetic medium required the addition of sodium bicarbonate for glutamyl polypeptide synthesis while 2 avirulent strains did not. Similar results were obtained with cells grown in liquid media (Thorne et al., 1953). The sporulation of Bacillus sphaericus was stimulated by the addition of bicarbonate or alpha-ketoglutarate to the medium (Powell and Hunter, 1955). Powell and Strange (1956) observed the accumulation of alpha-ketoglutarate in a complex medium before the onset of sporulation in B. cereus and B. subtilis.

As made evident by the literature, the nutritional environment is intimately associated with the biochemical mechanisms operating during growth and sporulation of aerobic bacilli.

MATERIALS AND METHODS

A strain of Bacillus cereus var. mycoides was used throughout this investigation. Growth and sporulation of the organism was usually accomplished by using a glutamate-salts liquid medium (MSG medium) which contained 1.7 percent L-glutamic acid sodium monohydrate (Mann Research Laboratories, New York), 0.02 percent $MgSO_4 \cdot 7H_2O$, 0.0005 percent $CaCl_2 \cdot 2H_2O$, 0.00001 percent $CuSO_4 \cdot 5H_2O$, 0.001 percent $MnSO_4 \cdot 5H_2O$, 0.001 percent $ZnSO_4 \cdot 5H_2O$, 0.00001 percent $FeSO_4 \cdot 5H_2O$, and 0.1 percent K_2HPO_4. The final pH of the medium was adjusted to pH 7.0-7.5 with 0.1 N-5.0 N HCL and/or NaOH. Modifications of this medium requiring other carbon and nitrogen sources are described elsewhere when necessary. All pH measurements were made with either a Model 76 Beckman expanded scale pH meter or a Model 3 Coleman pH Electrometer. All media were sterilized by autoclaving at 18 psi for 20 minutes except when 14 liter glass fermentors were used. Three glass fermentors containing 10 liters of medium were autoclaved together at 20 psi for 2 hours.

Both vegetative cells and heat-shocked spores were used for inoculation. When vegetative cells were used, the organisms were subcultured 3 times in the desired medium and incubated at 31°C on a variable speed Eberbach reciprocating shaker at 100-110 strokes per minute. Cells harvested prior to sporulation were washed aseptically 3 times in sterile deionized water. The inocula were pipetted from a suspension of sterile deionized water and cells. To obtain a spore inoculum the organism was cultured in 10 liters of MSG medium contained in a 14 liter fermentor. Sporulation was allowed to proceed to completion (95-100 percent). The

spores were harvested by centrifugation with a steam-driven Sharples Super-Centrifuge, washed 4 times in deionized water and acetone-dried according to the method given by Umbreit et al. (1959). Refrigeration of this preparation at 4°C provided a stable inoculum showing only a negligible decrease in viability over a 2 year period. This spore inoculum was quantitated by heat-shocking a weighed sample contained in a known volume of deionized water and then making plate counts on nutrient agar.

Heat-shocking was performed at 80°C for 30 minutes in a water bath to stimulate germination and to aid in eliminating contamination. Inoculation procedures using the spore inoculum consisted of heat-shocking a weighed sample of the acetone-dried spore preparation added to 5 ml of sterile deionized water contained in a sterile 16 x 125 mm screw capped test tube and pipetting the desired quantity into sterile media.

To test the effects of additives, generally 500 ml and 1000 ml of medium contained in 3 liter Fernback flasks and 6 liter Erlenmeyer flasks, respectively, were inoculated with heat-shocked spores which were allowed to germinate at 31°C while shaking. When outgrowth was assured, equivalent quantities of the inoculated media were distributed to sterile 250 ml growth flasks containing the additive. A tubular projection (30 ml capacity), sealed at one end and attached to the growth flask at the other, permitted prompt turbidimetric readings simply by tilting the flask, allowing the medium to run into the attached tube, and then placing the tube directly into the adaptor on the colorimeter. Growth of the organism in these flasks was followed turbidimetrically by recording the optical density (O.D.) at 420 or 625 millimicrons with a Bausch and Lomb

"Spectronic-20" colorimeter.

The growth of cells in fermentors was followed turbidimetrically with a Beckman Model 150 Spectro-colorimeter. With MSG medium a wavelength of 420 or 650 millimicrons was used. The use of glucose-yeast extract-salts medium required a wavelength of 650 millimicrons due to the yellow color of the medium.

Intact cells used for enzymatic studies were harvested from Erlenmeyer flasks containing liquid media by centrifugation in a Servall SS-1 centrifuge at 29,000 x g for 10 minutes after which they were washed 6 times with 0.10 M, pH 7.0, phosphate buffer. The cells were then re-suspended in phosphate buffer at the desired concentration. All phosphate buffers were prepared according to Lange (1956).

Cellular extracts for use in enzymatic studies were obtained from cells grown in 14 liter fermentors containing 10 liters of medium. A cell-buffer paste was shaken with small glass beads (about 0.2 mm diameter) in a Mickle tissue disintegrator at maximum amplitude until microscopic examination revealed 90-100 percent breakage. Cellular debris was removed by centrifuging at 4°C for 60 minutes in the Servall SS-1 centrifuge at approximately 35,000 x g, after which the supernatant was retained and diluted with buffer to a protein concentration of 4.0-7.0 milligrams per milliliter. A phosphate buffer of pH 7.4 was used as the diluent except when alpha-ketoglutarate carboxylation and isocitrate lyase measurements were desired, then a trihydroxy methyl amino methane (Tris) buffer of pH 7.2 was employed.

Protein determinations were performed according to the method

described by Lowry et al. (1951). The reagents required for this method are:

reagent A: 2 percent sodium carbonate in 0.1 N sodium hydroxide

reagent B: 1:1 ratio of B_1 (1.0 percent $CuSO_4 \cdot 5H_2O$) and B_2 (2 percent sodium potassium tartrate).

reagent C: 50 ml of reagent A plus 1 ml reagent B.

reagent D: 2 N phenol reagent (Folin-Ciocalteau) diluted 1:3.

Standard protein solution: bovine albumin (Armour) diluted so that 1 ml contained 0.1 mg.

Four ml of reagent C were added to individual 50 ml Erlenmeyer flasks containing 1 ml of appropriately diluted extract. This mixture was allowed to stand at room temperature for at least 10 minutes. Four-tenths ml of reagent D was added to each flask, mixed well and allowed to stand at room temperature for at least 30 minutes. The optical density of the sample flasks was measured against a water-reagent blank, treated in exactly the same way, at 650 millimicrons with the Beckman Model 150 Spectro-colorimeter. The protein concentrations were calculated from standards which had been treated in the same manner.

Dehydrogenases of cellular extracts were measured by the Thunberg technique as described by Umbreit et al. (1959). Closely calibrated Thunberg tubes each with a removable ground glass head assembly equipped with a permanent sidearm attachment were used. Two ml of pH 7.4 phosphate buffer and 1 ml of a 1:10 dilution of a stock concentration of methylene blue (0.1 percent weight/volume) were placed in the main body of the tube. One ml of cellular extract, diluted to a suitable protein concentration

with pH 7.4 phosphate buffer, was added to the sidearm. The ground glass of the head assembly was lubricated with a silicone lubricant (Dow Corning stopcock grease) and rotated until the small hole in the ground glass joint was lined up with a glass tube projecting from the main body of the tube. One end of a rubber pressure hose was attached to this glass tube while the other end was affixed to a Hyvac 7 vacuum pump. The tubes were evacuated for 3 minutes after which the head assembly was slowly turned 180 degrees to maintain in vacuo conditions. Each tube treated in this manner was incubated in a 31°C water bath and allowed to equilibrate for 30 minutes. The extract was then thoroughly mixed with the rest of the reaction mixture by inverting the tubes several times. In the absence of oxygen, methylene blue serves as electron acceptor becoming a colorless leuco compound upon reduction. The general reaction involved is as follows:

$$AH_2 + MB \xrightarrow{\text{dehydrogenase}} A + MBH_2$$

substrate	methylene blue	oxidized substrate	reduced methylene blue (colorless leuco compound)

Reactions of this nature were followed with the Beckman Model 150 Spectrocolorimeter by periodically recording the change in absorbancy at 650 millimicrons. Because an endogenous control containing an equal volume of deionized water instead of substrate was used as the reference, any activity contributed by endogenous dehydrogenation was automatically corrected for. Specific activity of dehydrogenases is defined as that amount of enzyme which caused a change in O.D. of 0.001/10 minutes/mg protein.

Alpha-ketoglutarate carboxylation was measured by following the oxidation of exogenously supplied reduced nicotinamide adenine dinucleotide phosphate (NADPH, formerly referred to as TPNH or TPNred) in the presence of sodium bicarbonate (previously flushed with 100 percent carbon dioxide for 30 minutes) at 340 millimicrons (UV) with the Beckman DB recording spectrophotometer (Ochoa, 1948). The reaction mixture in the experimental cell consisted of: 0.4 ml of 0.1 M glycine buffer, pH 7.2; 0.4 ml of 12 micromolar $MnCl_2$; 1.0 ml of 0.14 micromolar NADPH (Sigma Chemical Co., St. Louis, Missouri); 0.1 ml of 2000 micromolar alpha-ketoglutarate (Nutritional Biochemicals Corporation, Cleveland, Ohio); 0.6 ml of 0.1 M $NaHCO_3$; 0.5 ml of dialyzed cellular extract. Dialysis was performed at zero degrees centigrade for 12 hours against deionized water which was completely changed frequently. The reaction mixture was run against a reference sample with the same additions as the experimental cell with the exception of NADPH which was replaced by an equal volume of deionized water. Endogenous oxidation of NADPH was determined by substituting an equal volume of deionized water for alpha-ketoglutarate in the experimental cell. Specific activity, which again is defined as that amount of enzyme catalyzing an O.D. change of 0.001/10 minutes/mg protein, was determined from the data obtained with the test mixture corrected for endogenous NADPH oxidation.

Calibrated, dual sidearm Warburg reaction flasks of 16 ml capacity, containing a total liquid volume of 3.0 ml, were used in all manometric measurements. All manometric measurements were performed in a 31°C water bath while shaking at 108 strokes/minutes. A thermobarometer consisting

of a reaction flask containing 3.0 ml of deionized water was used to correct for changes in temperature and atmospheric pressure during each experiment.

Oxygen uptake was determined manometrically. The main chamber of each test flask contained 0.8 ml of 0.1 M phosphate buffer, pH 7.0 and 1.0 ml of 0.15 M substrate. The center well contained 0.2 ml of 20 percent KOH for carbon dioxide absorption and a small piece of fluted filter paper which provided a greater surface area. One specific sidearm of each flask was always empty while the other contained 1 ml of cells. Endogenous controls containing 1 ml of deionized water instead of substrate were used to account for endogenous activity. After allowing sufficient time for temperature equilibration, usually 20-30 minutes, the flasks, attached to manometers, were inverted several times to mix the reagents and readings were taken periodically.

Glyoxylate carboligase activity was determined by manometric measurement of CO_2 evolution (Umbreit et al., 1959). By employing 2 sets of flasks each respiring in the same manner, except that in 1 set the carbon dioxide was absorbed whereas in the other it was not, the CO_2 liberated could be measured. Each flask in which CO_2 was absorbed, and which measured oxygen uptake, contained 0.2 ml of 20 percent KOH in the center well. The main compartment received 1.0 ml of 0.025 M sodium glyoxylate monohydrate (Sigma Chemical Co., St. Louis, Missouri) and 0.8 ml of phosphate buffer, pH 6.4. One ml of cellular extract was placed in one of the sidearms. Each flask in which CO_2 was not to be absorbed contained 0.2 ml of deionized water in the center well instead of 20 percent KOH.

To account for endogenous CO_2 evolution, 2 sets of flasks, one to measure oxygen uptake and one to measure the release of carbon dioxide, contained 1.0 ml of deionized water instead of glyoxylate. Thus, all data reported are corrected for endogenous activity. The reagents in all flasks were mixed as previously described and readings were taken periodically. Computations were performed according to Umbreit et al. (1959). Specific activity is defined as microliters of carbon dioxide evolved per hour per milligram of protein.

Isocitrate lyase and malate synthetase measurements were conducted according to slight modifications of the methods given by Dixon and Kornberg (1959). The isocitrate lyase assay was modified only to the extent that DL-isocitrate, trisodium salt (Sigma Chemical Co., St. Louis, Missouri) was used as the substrate instead of potassium L_s-isocitrate. The principle of this assay depends on measuring the rate of increase of O.D. with the Beckman DB recording spectrophotometer at 325 millimicrons (UV) upon the formation of glyoxylate phenylhydrazone. Three ml of reaction mixture in the sample cell contained 100 micromoles of pH 7.2 Tris buffer, 15 micromoles $MgCl_2$, 10 micromoles pheylhydrazine·HCl (Mann Research Laboratories, New York, New York), 0.2 ml extract, 10 micromoles DL-isocitrate, and 0.3 ml deionized water. The reference cell contained the same reagents except for the substitution of an equal volume of deionized water for isocitrate.

The malate synthetase reaction was quantitatively adapted to the silica cuvettes used with the Beckman DB recording spectrophotometer. Four ml of 0.1 M Tris buffer, pH 8.0 were mixed with 0.15 ml of 0.1

M $MgCl_2$ and 0.1 ml of 0.002 M acetyl coenzyme A (Sigma Chemical Co., St. Louis, Missouri). Three ml of this mixture were added to the test cell along with 0.5 ml of extract. The reference cell contained 3 ml of a mixture containing 4.0 ml of 0.1 M pH 8.0 Tris buffer, 0.15 ml of 0.1 M $MgCl_2$, and 0.1 ml deionized water. To this was added 0.5 ml deionized water in place of the extract. No change in absorbancy at 232 millimicrons indicated the absence of acetyl coenzyme A deacylase, so 0.1 ml of glyoxylate was added as substrate to both the reference and test cells. No observable change in absorbancy was evident, denoting the absence of malate synthetase.

Glutamate decarboxylation was assayed by the same manometric techniques described for glyoxylate carboligase with L-glutamic acid (Mann Research Laboratories, New York, New York).

Qualitative studies of glutamate transamination were performed by one dimensional paper chromatography using as solvents 80 percent phenol and butanol-glacial acetic acid-water (4:1:1). A typical reaction mixture containing 0.5 ml of 0.08 M keto-acid, 0.02 micrograms of pyridoxal phosphate (Calbiochem, Los Angeles, California), and 1.0 ml of extract was allowed to incubate in a 31°C water bath for 60 minutes. To stop any reaction which might be proceeding, 1.0 ml of 15 percent trichloroacetic acid was added to each mixture. After centrifugation at 35,000 x g in the Servall SS-1 super centrifuge at 0°C, the supernatant of each test mixture was spotted on 8" x 10" sheets of Whatman #1 filter paper. Appropriate amino acid standards of 0.01 M concentration were spotted on the same sheets of filter paper. Spotting was done with broken wooden

applicator sticks. All chromatograms were run separately in the 2 solvents, dried in an oven at 80°C and developed with ninhydrin spray (triketohydrindene hydrate). Comparisons were made between reaction mixtures and their respective amino acid standards.

RESULTS

To initiate a physiological study of sporogenesis, it is desirable to develop the simplest chemically defined medium which supports adequate growth and maximum sporulation. The synthetic media used in investigations of aerobic spore formers are basically the same, varying principally in organic constitution (Roberts, 1934; Foster and Heiligman, 1949b; Grelet, 1946; Williams and Harper, 1951; Perry and Foster, 1955; Hanson et al., 1961).

Table 1 shows the effect of several media, differing in organic composition only, on the sporulation of B. cereus var. mycoides. The inocula were taken from flasks containing a third subculture obtained by growth in the medium to be tested. Of those examined, the best medium for complete sporulation of this particular organism was the MSG medium, which was actually the most elementary. Although media supplemented with glucose (Fisher Scientific Co., Fairlawn, New Jersey) gave higher growth yields, sporulation appeared to be somewhat incomplete, the sporulating cell retaining some of the sporangium. Figure 1 represents a standard curve showing growth and sporulation of B. cereus var. mycoides grown aerobically by shaking at 31°C in 250 ml culture flasks. Twenty-five ml of MSG medium were inoculated from MSG-agar slants and incubated for 21 hours at 31°C. A subculture was made by adding 0.1 ml of the initial culture to 25 ml of MSG medium and incubating until 100 percent sporulation was achieved. The spores were separated from the medium by centrifugation, washed 3 times in deionized water and finally resuspended in 5 ml of deionized water. This suspension was heat-shocked for 15

Table 1. Sporulation in media differing only in organic constitution

Organic constituents[a]	% sporulation[b] after 48 hours
Glucose (0.5%) monosodium glutamate (0.8%)	100[c]
Glucose (0.5%) asparagine (0.8%)[d]	--[e]
Succinate[f] (0.66%) asparagine (0.8%)	50
Monosodium glutamate (1.7%)	100
Succinate (0.66%) monosodium glutamate (0.8%)	90

[a]Supplemented with all inorganic constituents of basal medium, pH 7.2.

[b]At 31°C on reciprocating shaker (101 strokes/minute).

[c]Sporulation incomplete (spores with some sporangia still attached).

[d]Obtained from Pfanstiehl Chemical Co., Waukegan, Illinois.

[e]Only negligible sporulation observed.

[f]Obtained from Fisher Scientific Co., Fairlawn, New Jersey.

minutes at 80°C with subsequent additions of 1.0 ml to growth flasks containing 25 ml of MSG medium. The growth curve was determined by following turbidity, while sporulation was determined by plate counts of aliquots which had been heat-shocked for 15 minutes at 80°C.

In further evaluation of the consequences of medium constituents on sporulation, the effects of manganese were studied indirectly since sporulation requires a concentration of this ion in excess of that required for growth (Charney et al., 1951; Curran and Evans, 1954; Curran, 1957). A possible function of manganese in sporulation has been investigated.

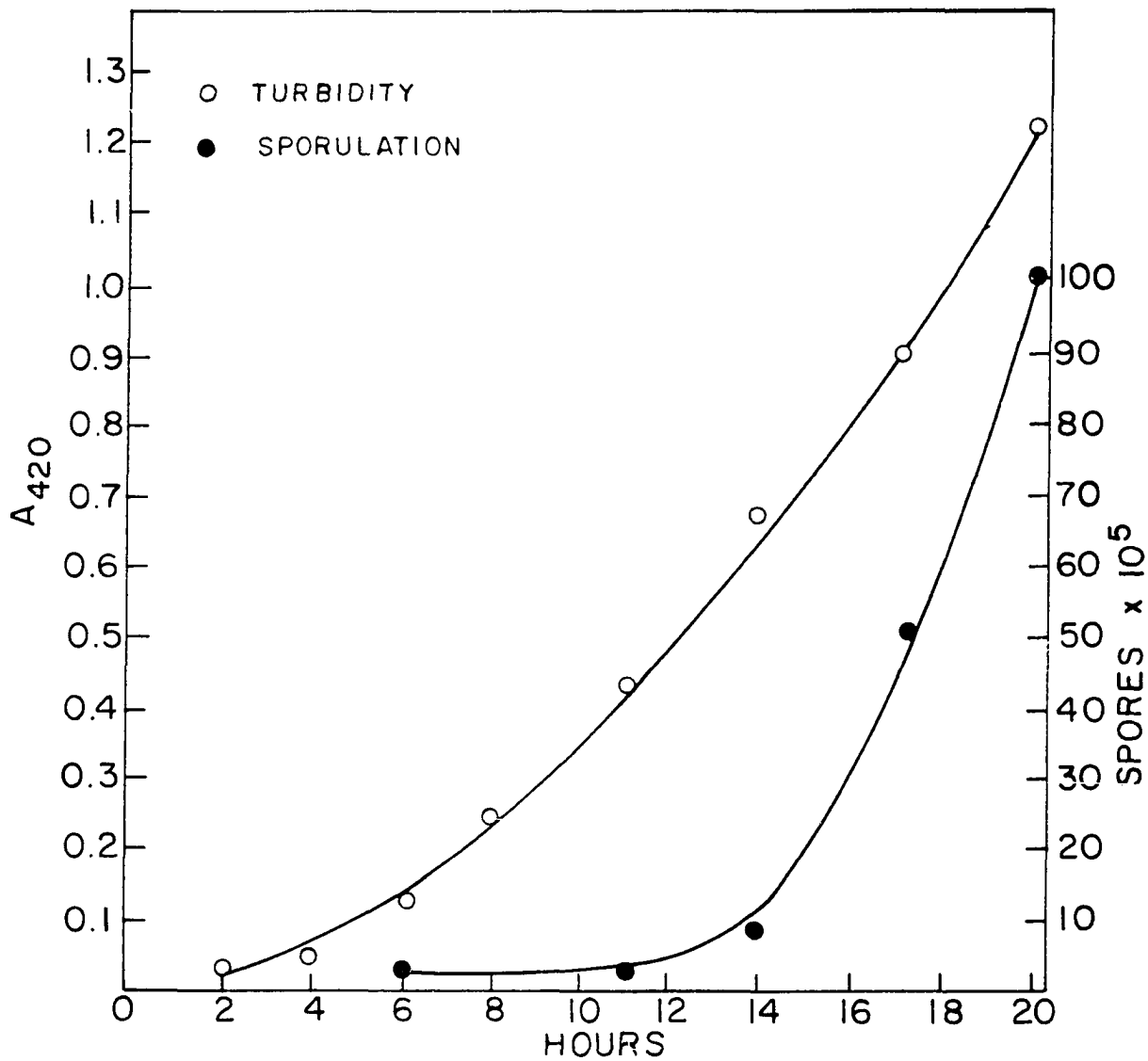

Figure 1. Standard growth and sporulation curves of Bacillus cereus var. mycoides grown in 25 ml of MSG medium contained in 250 ml flasks

Stockton and Wyss (1946) demonstrated a manganese-requiring proteinase in B. subtilis. Weinberg (1955) observed that manganese stimulation of sporulation in B. subtilis was ineffective if withheld more than 6 hours after the cessation of logarithmic growth. In an effort to determine at what stages the ion might be required for sporulation, chelation studies with kojic acid (Nutritional Biochemicals Corporation, Cleveland, Ohio) were conducted. Kojic acid has been shown to be an effective chelator of manganese (Mayer, 1962). The organism was cultured in MSG medium at 31°C for 18 hours on a Cutler-Hamner Model V rotary shaker after which the medium was distributed equally into four 250 ml culture flasks (50 ml/ flask). To 2 flasks kojic acid (final concentration of 1250 micrograms per milliliter) was added and to 2 others an equivalent amount of deionized water. The cultures were reincubated under the aforementioned conditions. Microscopic examinations of stained smears were made at intervals and the amount of sporulation estimated (Table 2). The high concentration of kojic acid appeared to halt growth in flasks containing the additive (kojic acid) which may account for the lack of sporulation in those flasks. Reducing the kojic acid level to 625 micrograms per milliliter (final concentration) and incorporating the additive at different times in the growth cycle still retarded growth somewhat (Figure 2). Table 3 shows the amount of sporulation at different times. When the level of kojic acid was reduced to 200 micrograms per milliliter (final concentration) in MSG medium, sporulation was not inhibited. However, when the same concentration of kojic acid was added to an autoclaved glucose-glutamate-salts medium, no sporulation was observed (Table 4).

Table 2. Effect of kojic acid (1250 μg/ml) on sporulation in MSG medium[a]

Time (hours)	% sporulation			
	With no kojic acid		With kojic acid	
	1a	2a	1b	2b
18	0	0	0	0
20	30	15	0	0
24	65	25	0	0
26	75	70	0	0
29	95	95	0	0

[a]Final concentration of kojic acid.

Table 3. Effect of kojic acid (625 μg/ml) on sporulation in MSG medium when added at different times during the growth cycle[a]

Time kojic acid added after inoculation	% sporulation		
	After 13.5 hours	After 19.5 hours	After 30 hours
None added	5	100	100
None added	5	100	100
4 hours	0	no growth	no growth
7 hours	0	0	0
10.5 hours	0	50	75
13.5 hours	0	95	98

[a]Final concentration of kojic acid.

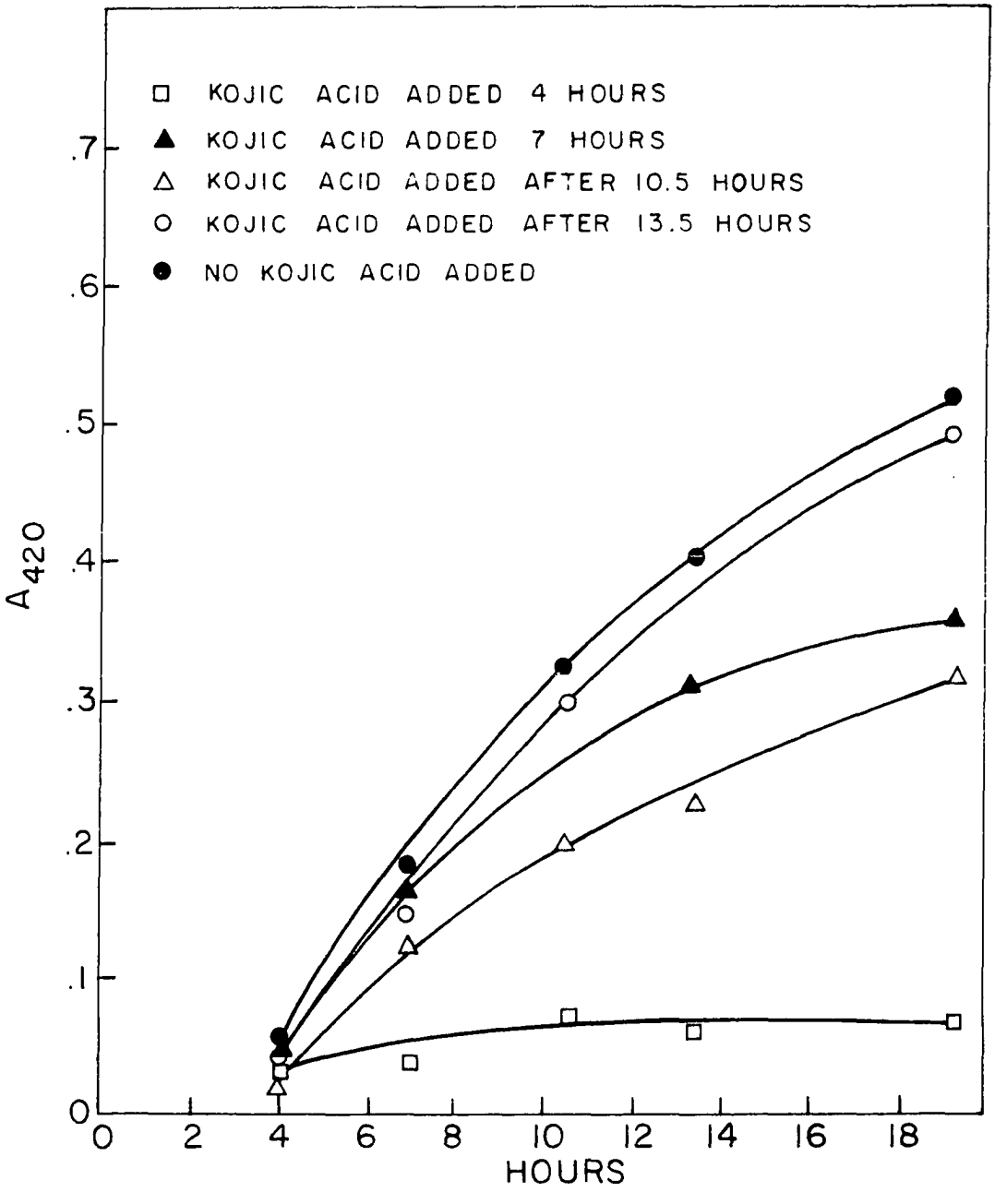

Figure 2. Growth rates in MSG medium due to additions of kojic acid (625 µg/ml, final concentration) at different times

Autoclaved glucose media yields an unidentified factor of suspected chelating potential (Traxler et al., 1962), thus the amount of actual chelating material in the glucose-glutamate-salts medium may have been more than was apparent, which may account for the decrease in sporulation because of less available manganese. On the other hand, the kojic acid concentration alone may not have been sufficient to chelate enough manganese to interfere with sporulation. These results indicate only that kojic acid in the proper concentrations can effectively inhibit sporulation up to a point (before commitment?) beyond which its effect is lessened. These data are presented only to show the initial approach to the problem. After many unsuccessful attempts to remove manganese from the medium so that critical concentrations of the ion required for growth and not sporulation could be determined, this line of attack was suspended in favor of other interests.

A growth medium containing only one organic compound as its sole source of carbon and nitrogen, as used in these investigations, is ideally suited for metabolic studies. Glutamate is a 5 carbon monoamino

Table 4. Effects of kojic acid on sporulation in 2 different media[a]

Medium	% sporulation after 48 hours
Glucose-glutamate	30
Glucose-glutamate-kojic acid	0
Glutamate	100
Glutamate-kojic acid	100

[a]Final concentration of kojic acid was 200 μg/ml.

dicarboxylic acid from which the organism must derive energy and carbon skeletons for biosynthesis. By studying the metabolism of glutamate and its intermediates at different stages of growth, it was hoped that enzymatic patterns leading to sporulation could be elucidated.

The most likely metabolic route for glutamate to take would seem to be through the TCA cycle via an initial dehydrogenation or transamination reaction to yield alpha-ketoglutarate. The alpha-ketoglutarate would subsequently be metabolized to succinate which in turn would be metabolized to fumarate via succinate dehydrogenase. Malonate has been shown to be an effective competitive inhibitor of succinate dehydrogenase (Potter and Dubois, 1943; Krebs et al., 1952). If glutamate normally proceeds through a succinate dehydrogenase system in this organism, then the addition of malonate to the medium in inhibitory concentrations (Krebs et al., 1952; Thorne, 1953) should show a physiological effect. Cultivation and inoculation procedures were performed as described in the section on materials and methods. Each flask contained 100 ml of medium to which had been added 1 ml of the desired sodium malonate (Eastman, Rochester, New York) concentration. MSG medium with no added malonate was used for controls. The unexpected results are shown in Figure 3. It may be observed that malonate had little or no effect on growth at all concentrations while sporulation was retarded only with the lowest level of malonate tested (Table 5). When the same concentration of malonate (0.1 M) was added at different stages of growth, the additive showed a significant stimulation of sporulation when compared with the control (Figure 4). The possibility that malonate alone might be used as a growth

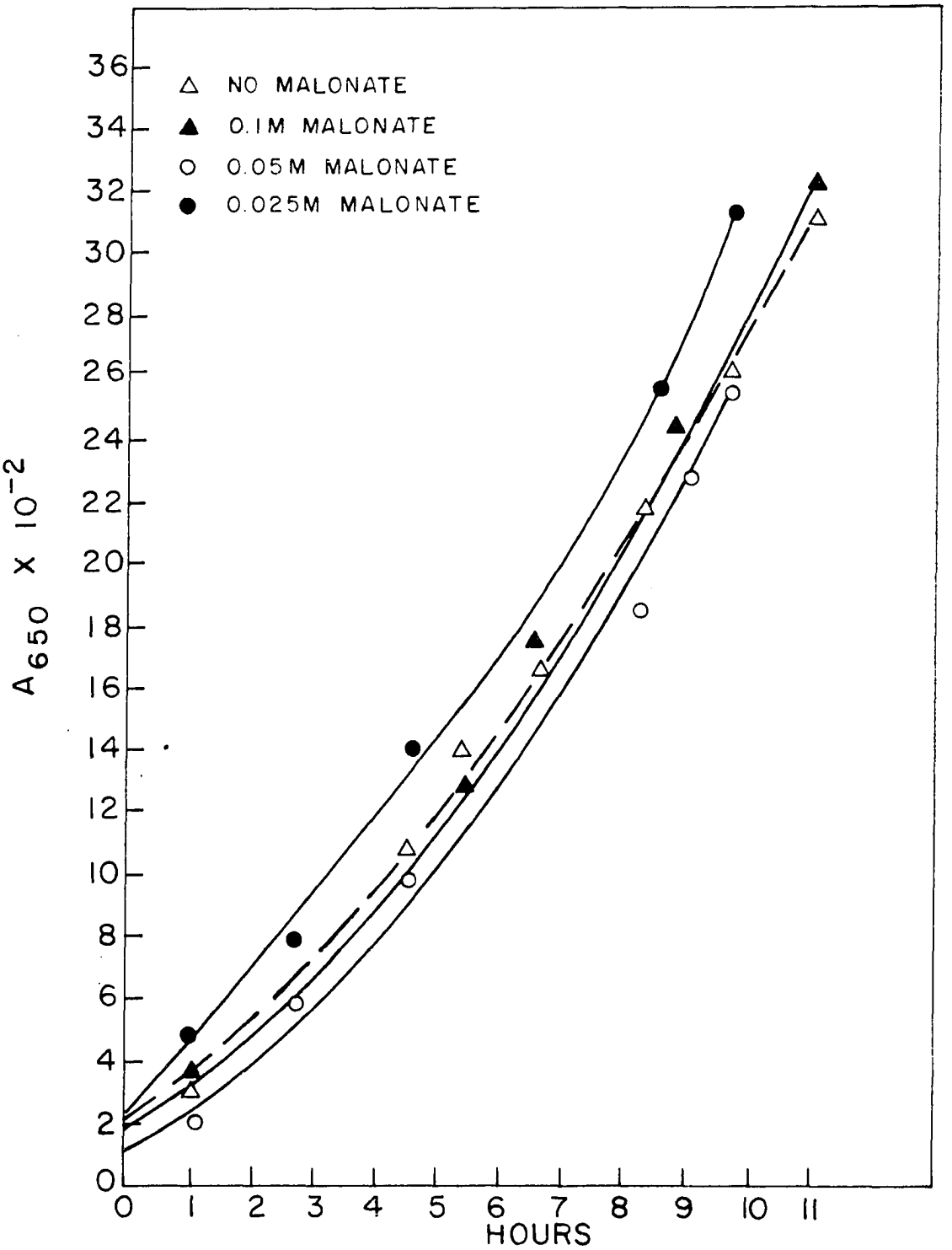

Figure 3. Growth of Bacillus cereus var. mycoides in MSG medium containing different concentrations of sodium malonate

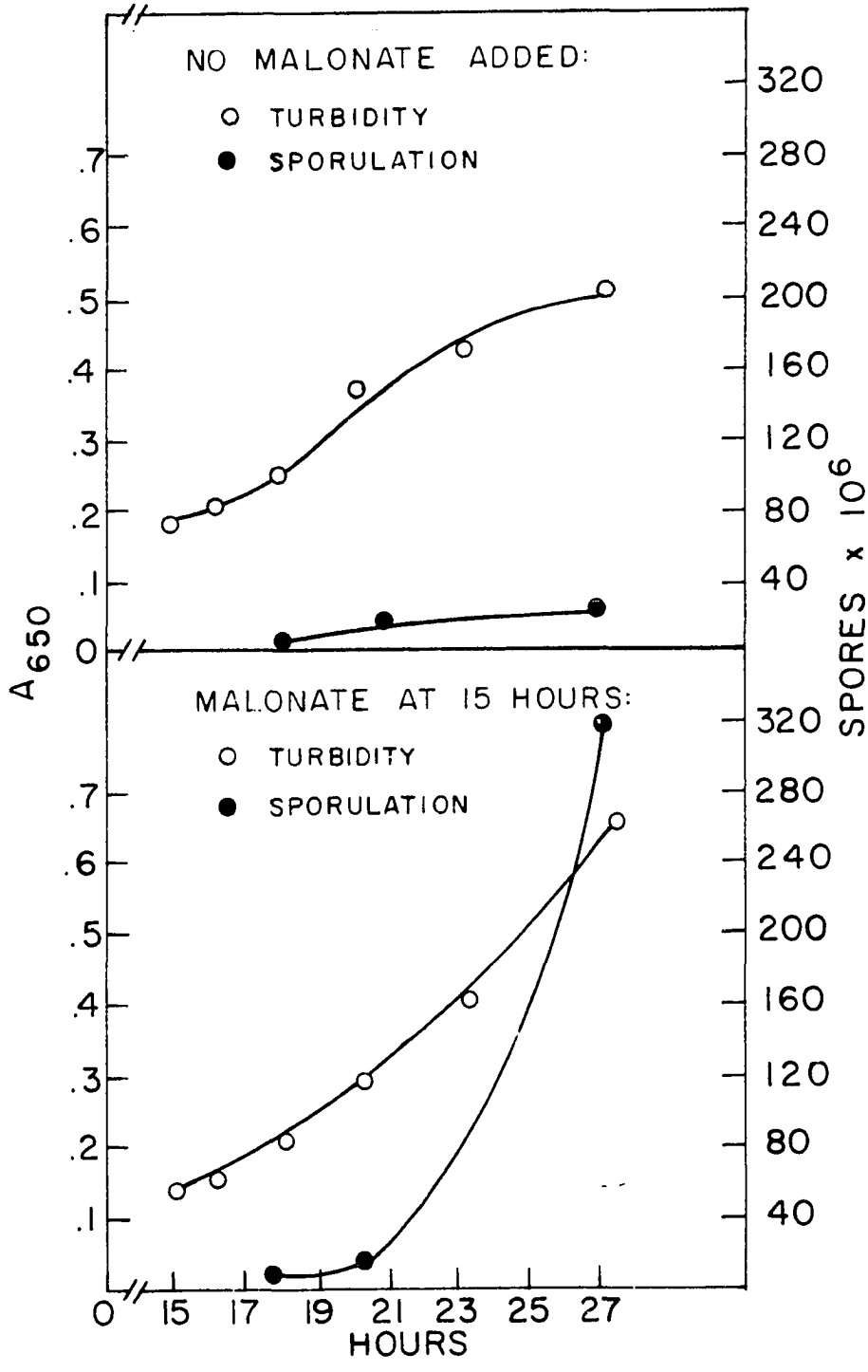

Figure 4. Stimulation of sporulation of Bacillus cereus var. mycoides by sodium malonate added during logarithmic growth

Table 5. Different malonate concentrations and their effect on sporulation in MSG medium[a]

Malonate concentration (molarity)	% sporulation after 31 hours
.1	90-100
.05	90-100
.025	15- 20
0	90-100

[a]Malonate concentrations added before inoculation and are final concentrations.

substrate by this organism was excluded when no growth resulted when malonate was substituted for glutamate in the basal medium supplemented with ammonium sulfate as the nitrogen source. This does not, however, eliminate the potential metabolism of malonate by enzymes not involved in growth.

Beevers et al. (1952) subscribe to the use of diethyl malonate instead of malonic acid for succinate dehydrogenase inhibition because of its greater permeability to the cell at near neutral pH. Table 6 reveals the results of using diethyl malonate (0.1 M) in MSG medium inoculated and cultured by the same procedures employed in the foregoing studies with sodium malonate. Since the diethyl malonate used was liquid (Eastman, Rochester, New York), an equivalent volume of deionized water was added to control flasks of MSG medium without the malonate ester. Sporulation, determined by examining stained smears after an incubation period of 30

Table 6. Effects of diethyl malonate on sporulation in MSG medium[a]

Medium	pH of medium after 30 hours	% sporulation after 30 hours
MSG	8.0	80-90
MSG - diethyl malonate	8.1	80-90

[a]Final concentration of diethyl malonate was same as glutamate (0.1 M).

hours, in the MSG-diethyl malonate medium was the equivalent of that observed in the MSG medium. Turbidity of the 2 media after 30 hours was comparable. However, it should be pointed out that diethyl malonate is rather immiscible with MSG medium making turbidity measurements difficult. Because of this property, the solubility of the ester may not have been great enough to provide an inhibitory concentration. R. E. Marquis, Department of Zoology, University of Edinburgh, Scotland, (1963, personal communication) suggested inoculating into a medium acidified to approximately the pK of malonic acid (about 5.0) and then neutralizing the medium following about 1 to 2 hours of shaking. Since malonate is more permeable to the cell in the non-ionized state, because it is more readily associated with the lipid portion of the cellular membrane in this form, the lower pH would permit greater permeability. Subsequent neutralization of the medium, while certainly providing better conditions for growth, might also prevent a "leaching out" of the absorbed malonate. Inoculated MSG medium adjusted to a pH of 5.0 with 1.0 N HCl in the presence of sodium

malonate (0.1 M) was allowed to shake for 1.5 hours on the reciprocating shaker at 31°C. The medium was then adjusted to pH 7.5 with sterile potassium hydroxide (1.0 N), dispensed aseptically into culture flasks (25 ml per flask) and reincubated at 31°C on the shaker. For controls MSG medium without malonate was similarly treated. Table 7 shows that

Table 7. Effects of altering the pH of MSG-malonate medium to allow greater malonate permeability

Medium[a]	pH of medium after 23 hours	% sporulation after 23 hours
MSG-malonate[b]	7.95	90
MSG	7.95	90

[a]Adjusted to pH 5.0 with 1.0 N HCl, allowed to shake for 1.5 hours on reciprocating shaker at 31°C and subsequently adjusted to pH 7.5 with sterile 1.0 N KOH.

[b]Final concentration of sodium malonate same as that of glutamate (0.1M).

sodium malonate at this concentration had no adverse effects on sporulation or change in pH.

Manometric techniques confirmed the presence of a weak but functional TCA system of enzymes (Table 8), which is in accord with other studies (Beck and Lindstrom, 1955). A comparison of MSG-grown cells with MSG-malonate-grown cells, both harvested at the approximate same time of late logarithmic growth and both adjusted to an O.D. of 0.1, revealed a definite inhibition of rates of oxygen uptake in the latter with alpha-

Table 8. A comparison of some oxidative enzymes of MSG-grown cells with MSG-malonate-grown cells[a]

Substrate	Q O_2	
	MSG-grown cells	MSG-malonate-grown cells
Glutamate	150.8	150.8
Alpha-ketoglutarate	50.8	31.0
Succinate	41.4	38.6
Isocitrate[b]	89.5	44.8
Glyoxylate[c]	50.5	36.7
Endogenous	35.5	17.8

[a]Cells harvested from late logarithmic phase of growth under standardized conditions (Figure 1).

[b]Initial velocity of reaction.

[c]Initial velocity of reaction.

ketoglutarate, isocitrate and glyoxylate as substrates. Of particular interest is that little or no effect was noted with the substrates glutamate and succinate. Oxygen uptake with isocitrate and glyoxylate proceeded initially at a constant rate but leveled off sharply after a short time.

To determine if similar effects on growth and sporulation could be observed with different carbon sources, malonate (final concentration, 0.1 M) was added to flasks containing the usual mineral salts but varying in carbon source only. All media contained 0.02 percent ammonium sulfate as a nitrogen source and 0.2 percent yeast extract (Difco, Detroit,

Michigan) which was required for growth in these media. Cultural conditions and inoculation procedures were those described in materials and methods. The results are summarized in Table 9. Malonate enhanced the growth rate in glucose-yeast extract medium, but increased lag and completely inhibited sporulation. In the acetate-yeast extract medium malonate showed some retardation of the lag phase and growth rate and completely inhibited sporulation. The growth rate in succinate was not suppressed by malonate but was actually stimulated although the lag was somewhat increased. No sporulation was observed in the succinate-yeast extract-malonate medium. Sporulation was not determined in succinate-yeast extract due to an oversight. The heat-shocked spore inoculum apparently had difficulty germinating in the asparagine-yeast extract medium, but the addition of malonate appeared to stimulate outgrowth although no sporulation was observed. Yeast extract, when incorporated into the mineral salts medium, served as an excellent growth substrate yielding good growth and sporulation even in the presence of malonate. Consequently, the full significance of these results is partially obscured by the necessity of using the ill defined yeast extract in these media as it is not clear whether the organism was utilizing the added carbon source or the yeast extract only. These results establish only that, assuming the added carbon sources were being utilized, although growth is not significantly affected by malonate in media containing glucose, acetate, or succinate, the metabolic pathways leading to sporulation in these media must proceed to a common point, that is, through the succinate dehydrogenase system. Bernlohr and Novelli (1960) showed that

Table 9. Effect of malonate on growth and sporulation in different media[a]

Medium	Lag (hours)	Growth rate[b]	% sporulation after 24 hours[c]
Glucose-yeast extract	4.10	36.0	100[d]
Glucose-yeast extract-malonate	5.00	42.5	0
Acetate[e]-yeast extract	4.30	43.0	100
Acetate-yeast extract-malonate	5.15	35.5	0
Succinate-yeast extract	2.75	20.0	-
Succinate-yeast extract-malonate	3.65	22.5	0
Asparagine-yeast extract	8 plus	slight	75
Asparagine-yeast extract-malonate	5.10	40.0	0
Yeast extract	3.10	27.7	100
Yeast extract-malonate	5.40	21.3	95

[a]Media supplemented with all inorganic constituents of basal medium and 0.02 percent ammonium sulfate; pH 7.2.

[b]Determined by slope of logarithmic growth.

[c]After incubation at 31°C on reciprocating shaker (103 strokes/minute).

[d]Incomplete sporulation with some sporangia still attached.

[e]As potassium salt from Mallinckrodt Chemical Works, St. Louis, Missouri.

ethyl malonate inhibited the sporulation of Bacillus licheniformis in a glucose salts medium. This is contrary to what was observed with the MSG medium in which malonate showed no inhibition of sporulation when added to the medium before inoculation.

Nakata and Halvorson (1960) observed an accumulation of organic acids during the growth of B. cereus T in a glucose-salts medium. They suggested that these acids, resulting from glucose degradation, served as substrates for sporulation. Results of Hanson et al. (1961, 1963a) indicate that acetate accumulation is due to the lack of the condensing enzyme in vegetative cells of B. cereus T. The condensing enzyme has been shown to be present and functional in extracts of cells harvested during early sporulation (Hanson et al., 1963b). If either the TCA cycle or glyoxylate cycle is involved in a physiological process, then that process should be inhibited by an adequate concentration of fluoroacetate. Inhibition of the TCA cycle is brought about by fluoroacetate, as fluoroacetyl Co A, condensing with oxaloacetate via condensing enzyme to form fluorocitrate, thereby inhibiting aconitase action (Brady, 1955; Marcus and Elliott, 1956). The glyoxylate cycle may be inhibited by the condensation of fluoroacetyl Co A and glyoxylate via malate synthetase to form fluoro-malate, thereby inhibiting malate oxidation. Studies conducted with B. cereus var. mycoides in MSG medium show that fluoroacetate (Calbiochem, Los Angeles, California) definitely inhibits both growth and sporulation when added before the middle of the logarithmic phase of growth, but later additions are less inhibitory (Table 10). Contrary to the observations of Hanson et al. (1961, 1963a), these data indicate an acetate condensing

Table 10. Effects of fluoroacetate on growth and sporulation in MSG medium when added at different times[a]

Time of fluoroacetate[b] addition after inoculation (hours)	O.D. after 21 hours	% sporulation after 17 hours	% sporulation after 31 hours
0	.084	0	0
2	.064	0	0
3	.078	0	0
10	.114	0	0
14.5	.176	0	20
17	.242	10	95-100
No fluoroacetate	.216	10	100

[a]Cultural conditions: 300 ml MSG medium contained in a 3 liter Fernbach flask was inoculated with 6.6×10^6 viable spores and incubated at 31°C on a reciprocating shaker for 2 hours, after which the inoculated medium was distributed to 250 ml culture flasks (25 ml per flask).

[b]Final concentration of fluoroacetate was 0.0076 M.

system(s) essential to the growth of early vegetative cells. The diminishing inhibitory effect of fluoroacetate when added at times after inoculation could be due to a shift in metabolic pathways as growth progresses, the excretion of a compound or compounds into the medium which overcomes the inhibition, or a combination of both. Working with Escherichia coli, Mager et al. (1955) found an excretory product which overcame the inhibitory effects of fluoroacetate. This excretory product was not identified. An accumulation of citrate could overcome the

inhibition by fluoroacetate. A similar study was conducted with B. cereus var. mycoides inoculated into log culture filtrates of cells grown in MSG medium (Table 11). Five hundred ml of MSG filtrate, obtained by separation from cells in the logarithmic phase of growth, were distributed into culture flasks containing filtrate, filtrate and fluoroacetate, and MSG medium. The flasks were then inoculated with heat-shocked spores and checked for sporulation after incubating for 23 hours at 31°C on the

Table 11. Effect of fluoroacetate added at time of inoculation on growth and sporulation in log culture MSG filtrate[a]

Medium	O.D. after 23 hours	Sporulation after 23 hours
MSG filtrate[b]	.260	+
MSG filtrate	.268	++
MSG filtrate + fluoroacetate[c]	.276	+++
MSG filtrate + fluoroacetate	.237	+++
MSG	.300	++++
MSG	.523	++++
MSG + fluoroacetate	.071	0
MSG + fluoroacetate	.187	0

[a]Cultural conditions: 1.9×10^6 viable spores added to flasks each containing 20 ml medium and incubated at 31°C on reciprocating shaker.

[b]Filtrate used with no supplementation.

[c]All final concentrations of fluoroacetate were 0.0076 M.

reciprocating shaker. These data show that fluoroacetate hardly inhibits growth, and in some cases stimulates sporulation in the filtrate. It should be pointed out that these results could not be repeated (Nancy Jo Bethea, Iowa State University, Ames, Iowa, 1963, personal communication). It is conceivable that high quantities of poly-beta-hydroxybutyrate excreted into the medium could surmount the inhibition ordinarily imposed by fluoroacetate in the quantities used.

Sporulation of Bacillus sphaericus in a casein hydrolysate medium was stimulated by the addition of bicarbonate or alpha-ketoglutarate (Powell and Hunter, 1955). The presence of carbon dioxide appeared essential to make sporulation rapid and complete. These investigators suggested that this may be true of sporulating organisms in general, since it has been repeatedly observed that rapid and complete sporulation occurs after a period of active growth. The addition of potassium bicarbonate (J. T. Baker Chemical Co., Philadelphia, Pennsylvania) to a slightly alkaline MSG medium (pH 7.9) stimulated the sporulation of B. cereus var. mycoides (Table 12). The inhibitory effect of a combination of malonate and bicarbonate is not understood.

Cantino and Horenstein (1956) reported an irreversible transition of Blastocladiella to a thick-walled, resistant sporangium which could be induced by adding bicarbonate to the growth medium. These workers suggested that the addition of bicarbonate reversed one or more steps in the TCA cycle, which led them to propose a reductive(?) carboxylation of alpha-ketoglutarate to form isocitrate. Since glutamate metabolism of B. cereus var. mycoides grown in MSG medium does not appear to be

Table 12. Effects of bicarbonate on sporulation in MSG and MSG-malonate media[a]

Medium[b]	% sporulation after 7 hours
MSG-malonate	0
MSG-$KHCO_3$	95
MSG	5
MSG-malonate-$KHCO_3$	no growth

[a]Cultural conditions: 31°C on reciprocating shaker; pH 7.9; inoculum, 1 x 10^6 viable spores inoculated into 25 ml medium.

[b]Final concentrations: $KHCO_3$ = 2.2 x 10^{-2} M, malonate = 0.1 M, monosodium glutamate = 0.1 M.

metabolized through relatively conventional pathways and because of the similarity of the transition in Blastocladiella and bacterial sporulation, the possibility of the carboxylation of alpha-ketoglutarate must be considered. Figure 5 illustrates this reaction occurring in dialyzed extracts of nonsporulating cells grown in MSG medium. The failure of potassium cyanide to inhibit endogenous oxidation of NADPH suggests either the reduction of some endogenous substrate or an electron transport system bypassing the cytochrome system, possibly an NADPH oxidase. The measurement of the reaction by the method employed is dependent on the reduction of the carboxylated product, oxalosuccinate, by NADPH to form isocitrate, showing that the reaction sequence proceeds at least to isocitrate. Barban and Ajl (1953) reported such a reaction occurring in Escherichia freundii.

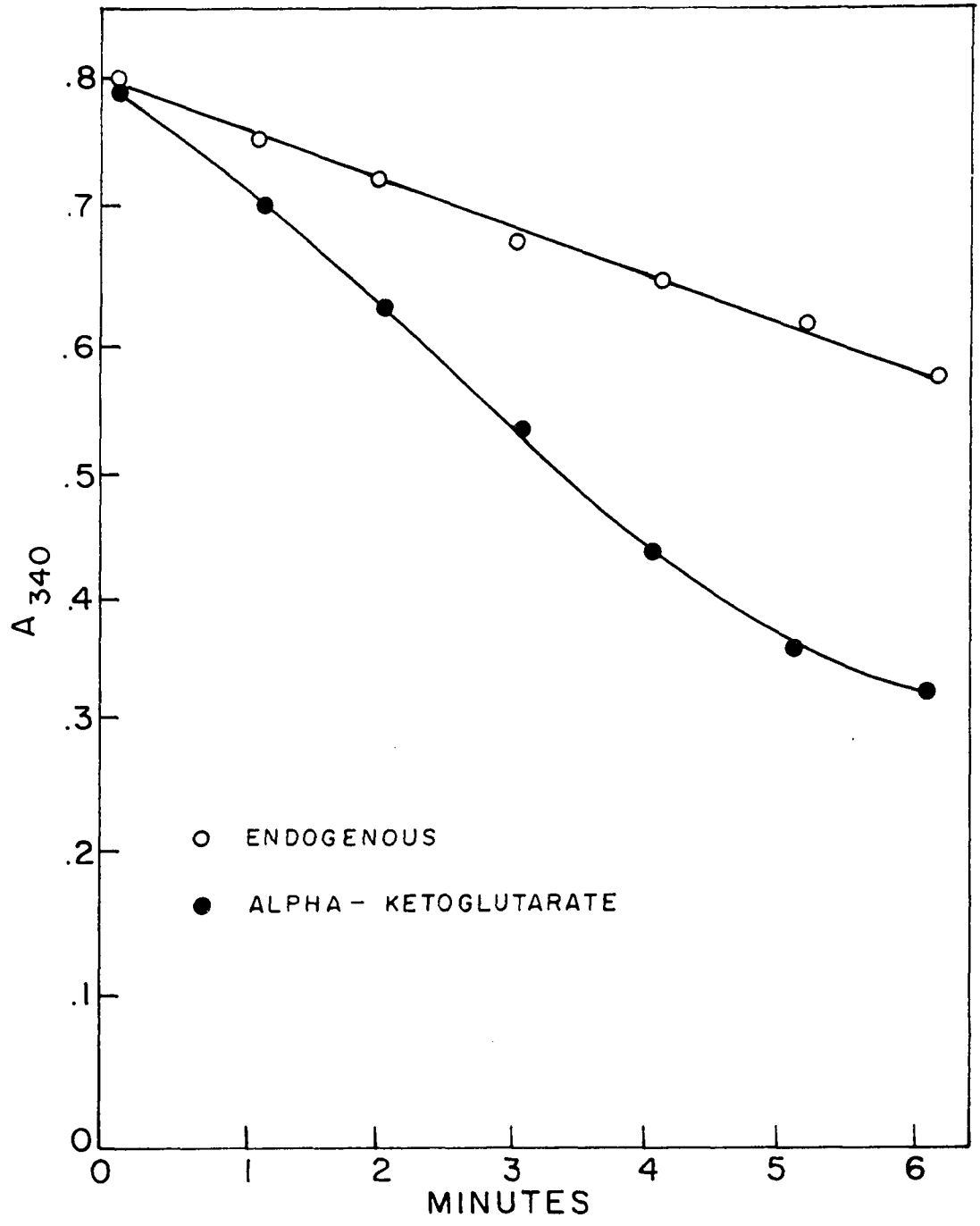

Figure 5. Carboxylation of alpha-ketoglutarate by Bacillus cereus var. mycoides harvested during early logarithmic growth

Because growth and sporulation of B. cereus var. mycoides in MSG medium apparently involve rather unique pathways of glutamate utilization, the necessity of comparing the metabolism of cells in early logarithmic growth with those of late logarithmic growth (during sporogenesis) was manifest. Although vegetative growth in MSG medium was not as plentiful as in other media, a sufficient quantity of cells in early logarithmic growth was obtained for the preparation of cellular extracts by harvesting 20 to 30 liters of inoculated medium. A standard growth curve (Figure 6) was determined by inoculating 10 liters of MSG medium, contained in a glass fermentation vat (capacity of 14 liters) with a known quantity of heat-shocked viable spores and measuring growth by recording turbidity at 1 and 2 hour intervals with a Beckman/Spinco Model 151 Spectro-colorimeter at a wavelength of 420 millimicrons. To assure maximal accuracy, all subsequent fermentation vats used for growing the organisms were accorded the same treatment as the vessel from which the standard curve was determined, including: inoculation procedures (weighed samples of acetone-dried spores were heat-shocked for 30 minutes at 80°C and the desired quantity pipetted into the fermentation vessel), autoclaving (2 hours at 20 psi), incubation temperature (31°C), impeller rotation speed (130 rpm), height distribution of the impellers (first impeller, 10 inches from top; second impeller, 15 inches from top) and the rate of aeration (0.06 volumes air/volume medium/minute). Although sporulation was less uniform using the aeration rates employed for the standard curve, higher rates of flow resulted in a more rapid rate of sporulation, thus less vegetative growth.

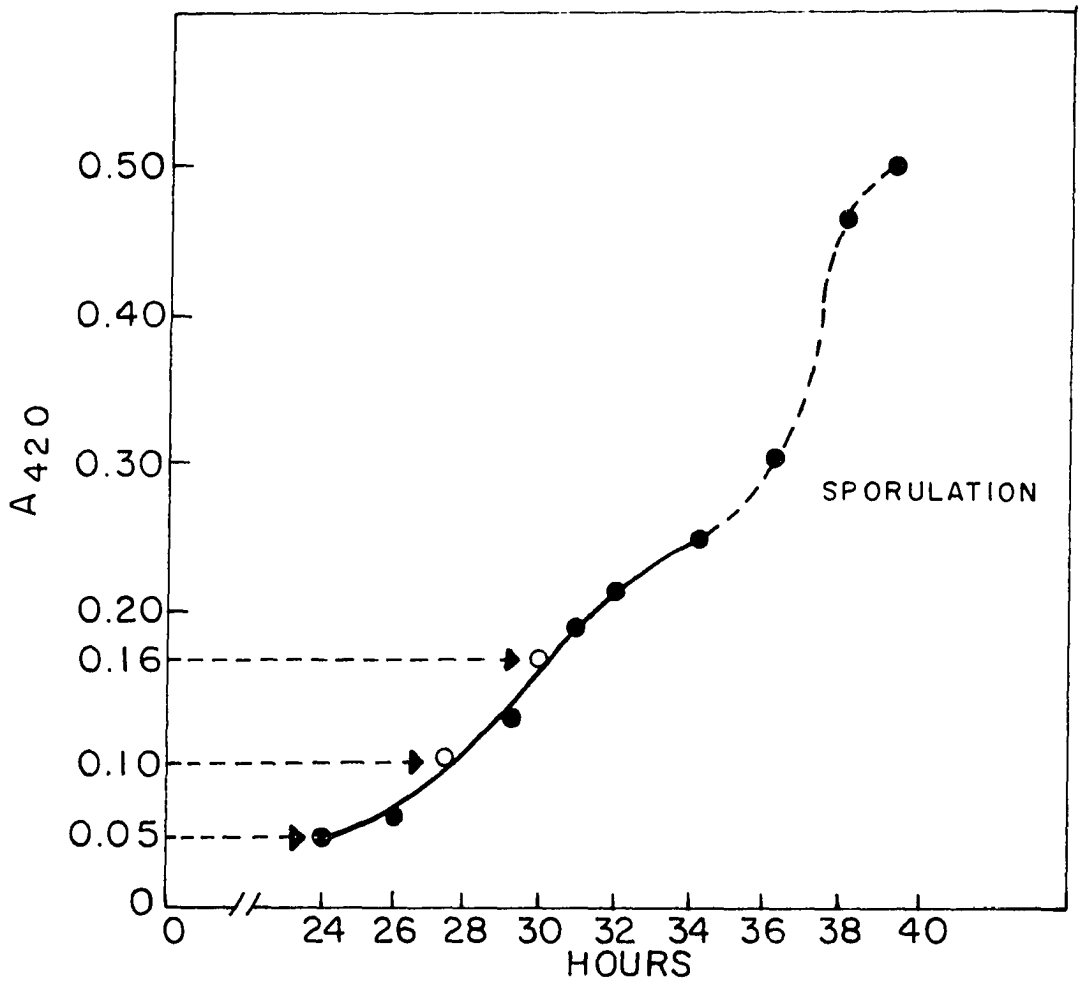

Figure 6. Standard growth curves of Bacillus cereus var. mycoides grown in 10 liters of MSG medium contained in a 14 liter fermentation vat

For enzymatic analyses, cells from 2 fermentors were harvested simultaneously at 3 different stages of growth, as indicated on the standard curve with MSG medium (Figure 6), within 1 hour. When 3 fermentors were used, the third was inoculated 1 to 2 hours later and harvested separately from the other two at the desired stage of growth. Procedures for washing cells, obtaining cellular extracts and measuring enzymatic activity are those described in materials and methods.

Definite metabolic shifts are shown by cells harvested from MSG medium at different stages of growth (Table 13). For the metabolic sequence from alpha-ketoglutarate to isocitrate to occur (reactions 2 and 4), isocitrate dehydrogenase must necessarily be functional, hence the measurement of the forward reaction of this enzyme reflects its ability to complete the reaction sequence. In cells harvested during early logarithmic growth (0.1 optical density) coupling of the NADPH generated from an active glutamate dehydrogenase (reaction 1) with the isocitrate dehydrogenase would supply the reduced pyridine nucleotide required to carry out the reduction of oxalosuccinate. The failure to detect glutamate dehydrogenase activity in these cells may be attributed to this coupling, which would be permitted by the relatively low alpha-ketoglutarate dehydrogenase activity (reaction 3). Since studies with fluoroacetate indicate a metabolic pathway leading to the formation of acetate, the presence of glyoxylate carboligase (reaction 5) would provide acetate for the suggested acetate condensing system and also the carbon dioxide necessary for the continuity of alpha-ketoglutarate carboxylation. It was thought that this acetate condensing system could be malate

Table 13. Specific activities of some enzymes during growth and sporogenesis of B. cereus var. mycoides in MSG medium

Enzymes	Specific activities		
	.05[a]	.10[b]	.16[c]
Glutamate dehydrogenase (reaction 1)	0	0	85.7
Enzyme(s) involved in alpha-ketoglutarate carboxylation (reaction 2)	0	135.8	17.2
Alpha-ketoglutarate dehydrogenase (reaction 3)	30.8	29.0	185.0
Isocitrate dehydrogenase (reaction 4)	615	294	0
Glyoxylate carboligase (reaction 5)	2.69	1.59	0.91

[a]Stage of late germination or early outgrowth.

[b]Early logarithmic phase of growth.

[c]Late logarithmic phase of growth.

synthetase since malate, as well as acetate, overcame the inhibition by fluoroacetate. However, no malate synthetase activity could be detected, by the method used, in cells harvested during early logarithmic growth in the MSG medium. The individual reactions mediated by the enzymes referred to in Table 13 are illustrated below:

glutamate + NADP ---------> alpha-ketoglutarate + NADPH (1)

alpha-ketoglutarate + CO_2 ----------> oxalosuccinate (2)

alpha-ketoglutarate + FAD + Co A --------> succinyl Co A + FADH (3)

oxalosuccinate + NADPH ----------> isocitrate + NADP (4)

glyoxylate + glyoxylate + TPP -------> tartronic semialdehyde + CO_2 (5)

Cells harvested during late logarithmic growth (0.16 optical density) show drastic changes in the activities of these enzymes. The increased alpha-ketoglutarate dehydrogenase activity at this stage of growth permits the detection of glutamate dehydrogenase because less alpha-ketoglutarate is available for carboxylation due to this enzymatic competition, thus the reactions involving alpha-ketoglutarate carboxylation, isocitrate dehydrogenase and glyoxylate carboligase show a marked decrease in activity.

Cells undergoing initial outgrowth ("physiological youth stage", 0.05 optical density) behave in a manner which is not commensurable with cells in early logarithmic growth.

Glutamate decarboxylase activity was absent in extracts of cells harvested during early or late logarithmic growth. Transamination between glutamate and oxaloacetate (Calbiochem, Los Angeles, California), glutamate and pyruvate (Calbiochem, Los Angeles, California), and glutamate and glyoxylate was not observed in extracts of cells harvested at an O.D. of 0.05 or during the late logarithmic phase of growth.

From the data thus far presented, it may be proposed that glutamate metabolism in early vegetative cells of B. cereus var. mycoides grown in MSG medium proceeds according to the scheme represented by Figure 7 (Beers and Mayer, 1963). As the cells are directed toward sporulation, an increased alpha-ketoglutarate dehydrogenase reaction shifts the metabolism

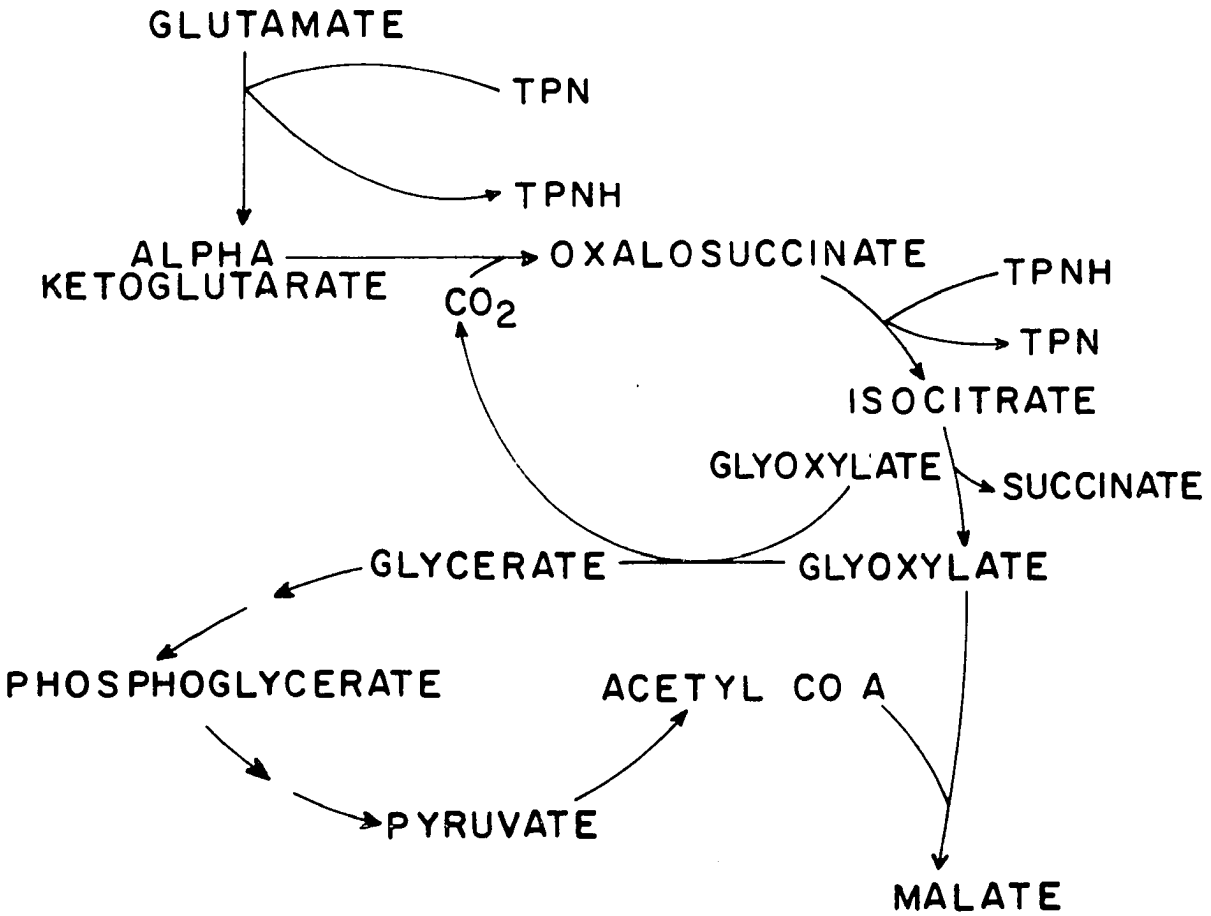

Figure 7. Proposed route of glutamate metabolism in cells of Bacillus cereus var. mycoides during early logarithmic growth

of the alpha-ketoglutarate formed via glutamate dehydrogenase to the formation of succinate.

The activities of glutamate dehydrogenase, alpha-ketoglutarate dehydrogenase, succinate dehydrogenase and isocitrate dehydrogenase were determined in cells harvested during early (0.26 optical density) and late (0.60 optical density) logarithmic growth in a glucose-yeast extract-salts medium (Table 14). The stages of growth at which cells were harvested in this medium were comparable to those in MSG medium, as made evident by a standard growth curve (Figure 8) determined in the manner described for the MSG medium. Cells were harvested during early logarithmic growth (optical density of 0.26) and during late logarithmic growth (optical density of 0.6) at the point where the pH of the medium began to rise. The

Table 14. Comparison of some TCA cycle enzymes in cells during early and late logarithmic growth in glucose-yeast extract-salts medium[a]

Growth phase[b]	Specific activities			
	Glutamate dehydrogenase	Alpha-ketoglutarate dehydrogenase	Succinate dehydrogenase	Isocitrate dehydrogenase
.26[c]	1.95	4.1	1.47	2.7
.60[d]	16.10	110.0	15.90	13.0

[a]Medium supplemented with all inorganic constituents of basal medium and 0.02 percent ammonium sulfate; pH 7.2.

[b]Cells harvested at stage of growth corresponding to standard growth curve (Figure 8).

[c]Early logarithmic phase of growth.

[d]Late logarithmic phase of growth.

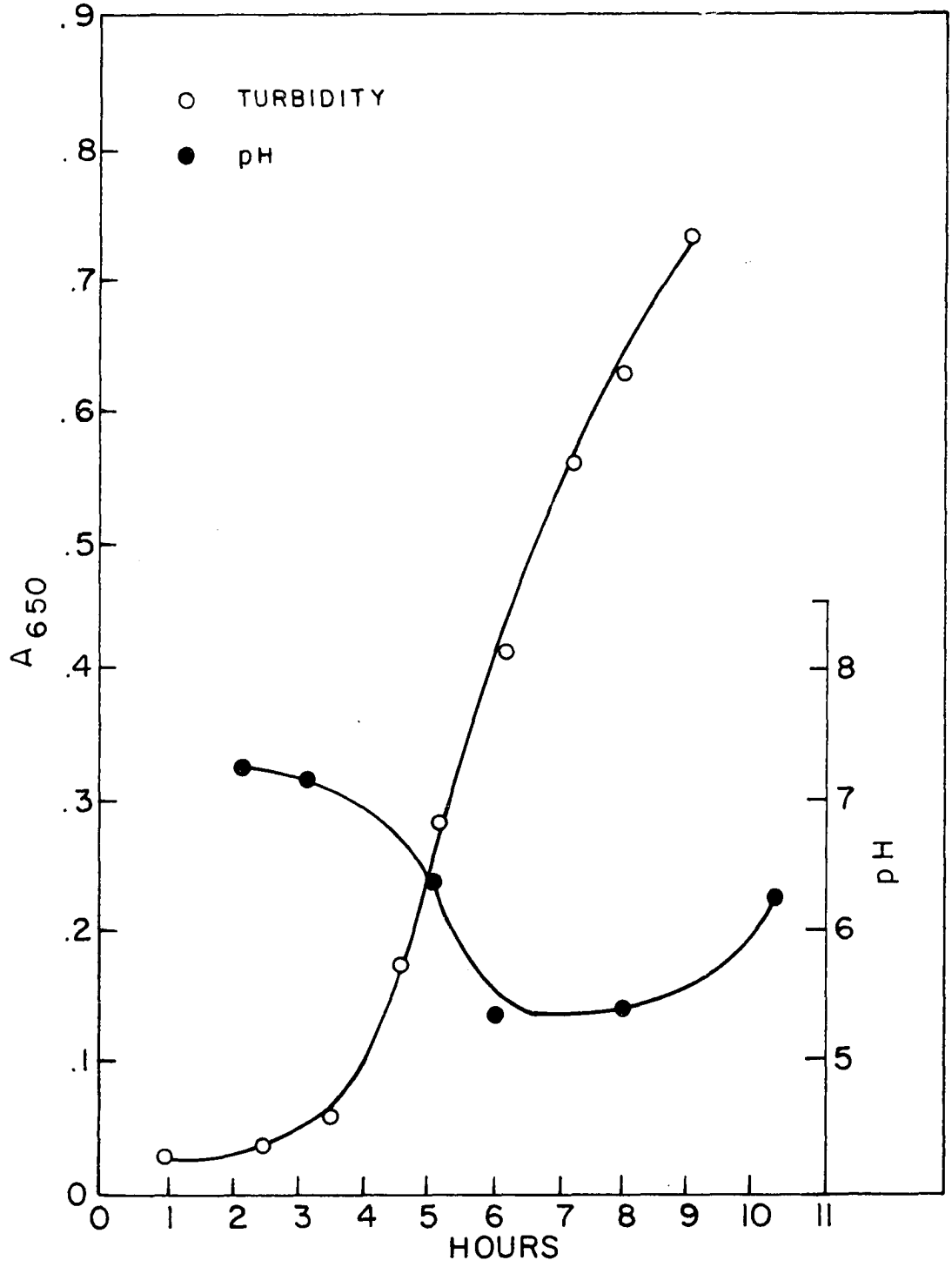

Figure 8. Standard growth and pH curves of Bacillus cereus var. mycoides grown in 10 liters of glucose-yeast extract-salts medium contained in a 14 liter fermentation vat

results indicate a shift to the TCA cycle in cells harvested during late logarithmic growth.

The activities of isocitrate lyase and succinate dehydrogenase were compared in cells harvested at a comparable early logarithmic stage of growth in MSG medium and glucose-yeast extract-salts medium (Table 15).

Table 15. Comparison of isocitrate lyase and succinate dehydrogenase in cells during early and late logarithmic growth in MSG medium and in glucose-yeast extract-salts medium

Medium	Growth[a] phase	Specific activities	
		Succinate dehydrogenase	Isocitrate lyase
MSG	Early log	0	19.4
	Late log	133.20	-
Glucose-yeast extract salts	Early log	1.47	0
	Late log	15.90	0

[a]Corresponds to respective standard curves (Figures 6 and 8).

The data show succinate dehydrogenase activity in both types of cells with the most pronounced activity in cells harvested from the MSG medium. Isocitrate lyase activity was demonstrated in cells grown in MSG medium but was nonexistent in cells harvested from the glucose-yeast extract-salts medium.

Hardwick and Foster (1952) showed that glucose inhibited sporulation if added before commitment. These workers concluded that the inhibitory

effect of glucose was due to its successful competition with the intracellular metabolism essential to sporogenesis. It was anticipated that cells grown in the MSG medium were committed to sporulation during early growth of the culture since in this medium the organism does not have to contend with a compound serving only as a growth substrate such as glucose. A comparison of the data obtained with cells harvested from the MSG medium at early stages of growth reveal changing metabolic patterns even before the middle of the logarithmic phase of growth. In further support of early commitment in MSG medium is the fact that much more vegetative growth can be obtained in glucose media before visible evidence of sporulation is observed. Cells grown in the MSG medium were harvested at different stages of growth (early log, 0.1 optical density and late log, 0.2 optical density), washed 3 times in phosphate buffer (0.1 N, pH 7.0) and resuspended in distilled water. Cells from both stages of growth were transferred to flasks containing glucose (0.3 percent) plus 5 ml deionized water and to flasks containing only 5 ml deionized water (controls). The flasks were shaken on the reciprocating shaker at 31°C (101 strokes/minute) and examined for sporulation at intervals. Those cells in which the enzymes necessary for sporulation have already been synthesized (committed cells) should sporulate in the presence of glucose. However, cells in which these enzymes have not yet been synthesized should sporulate much more slowly than the controls. Plate counts before and after sporulation, determined by heat-shocking the latter, revealed massive lysis of the vegetative cells (80-90 percent). The experiment was repeated using phosphate buffered saline (0.1 N phosphate buffer, pH

7.0; 0.85 percent saline solution) instead of deionized water, plate counts revealing only about 10-20 percent lysis. The results are shown in Table 16 and indicate that cells in the early stage of growth in MSG medium are committed to sporulation. Reference should be made to the fact that the lysis of vegetative cells to the extent of 10-20 percent

Table 16. Commitment of cells harvested from MSG medium during early and late logarithmic growth

Time (hours)[a]	Growth phase[b]	Phosphate buffer	Glucose + phosphate buffer[c]
0	0.1	0	0
	0.2	0	5
2	0.1	0	
	0.2	5	5
3	0.1	10	10
	0.2	30	35
4	0.1	80	75
	0.2	100	100
5	0.1	100	100
	0.2	-	-

[a]Time after transfer to phosphate buffer and glucose-phosphate buffer.

[b]Refers to optical density determined under standardized conditions (Figure 6).

[c]Glucose concentration was 0.3 percent; phosphate buffer was pH 7.0.

could, conceivably, contribute to precursor material for spore synthesis.

An effort was made to determine, enzymatically, the quantity of glutamate remaining in the medium after the initiation of sporulation in MSG medium; however, a cloudy precipitate developed upon addition of the enzyme (glutamate dehydrogenase, Nutritional Biochemical Corporation, Cleveland, Ohio) to the reaction mixture. Since the substrate in the reaction mixture was the glutamate in the medium presumably the precipitate resulted from the salting out of the protein due to the high ionic strength of the medium.

DISCUSSION

Many of the metabolic pathways leading to growth and sporulation resulting from the degradation and assimilation of dissimilar organic substrates will naturally be different; however, some of the biosynthetic reactions leading to sporogenesis are probably common to all spore formers.

In contrast to the conventional glucose-yeast extract-salts media usually employed in biochemical investigations of sporulating aerobic bacilli, the organism grown in MSG medium is initially committed to the metabolism of glutamate. The use of glutamate as the sole source of carbon and nitrogen eliminates the classical catabolic reactions of glucose that might otherwise mask important metabolic shifts involved in sporulation. In this simplest of chemically defined media in which sporulation has been studied, B. cereus var. mycoides must achieve the same end results were it grown in a more complex medium.

Glutamate metabolism by vegetative cells of B. cereus var. mycoides harvested during early logarithmic growth in MSG medium apparently proceeds first through a reaction involving glutamate dehydrogenase resulting in the formation of alpha-ketoglutarate. The product of this reaction is then reduced by the NADPH generated by glutamate dehydrogenase to form isocitrate.

The fact that malonate did not inhibit growth in MSG medium, glucose-yeast extract medium, or acetate medium, but did inhibit sporulation in the latter two, can be explained by the different pathways the organism takes during growth in the different media. Growth in MSG by B. cereus

var. mycoides is accomplished through a pathway yielding succinate from isocitrate via isocitrate lyase. Thus, if enough succinate is formed by the organism during the growth period, the inhibition imposed by malonate on succinate dehydrogenase could be overcome at a critical stage when this enzyme is needed, such as during sporogenesis. Growth of this organism in a glucose medium, and probably an acetate medium as well, does not involve succinate formation, at least not by way of isocitrate lyase. Hence, the succinate dehydrogenase reaction is blocked when it may be critically required for an exclusive gross synthesis, namely, spore material. Nakata and Halvorson (1960) and Hanson et al. (1963a) demonstrated the accumulation of acetate by B. cereus T resulting from glucose degradation and lack of condensing enzyme, consequently the glucose and acetate media may be considered quite similar. Yeast extract alone was shown to yield good growth and sporulation in the concentrations used, and since it was required by B. cereus var. mycoides for growth in both glucose and acetate media, the substrates for growth provided by this compound may be sufficient until condensing enzyme and other enzymes, such as alpha-ketoglutarate dehydrogenase and succinate dehydrogenase, become functional. The decreasing pH in the glucose-yeast extract medium conceivably could allow more malonate to enter the cell while far less malonate might enter the cell grown in MSG medium because of no significant drop in pH. Thus one could consider the effects of malonate in these media as a matter of permeability. However, two more lines of evidence, aside from those observations already discussed in the section on results, tend to minimize the significance of this surmise: 1) in the acetate-

yeast extract medium (initial pH, 7.2) no significant drop in pH was noted during growth of B. cereus T (Megraw, 1964) or B. cereus var. mycoides, and the inhibitory effects of malonate, especially the inhibition of sporulation, compared favorably with those observed with the same organism grown in the glucose-yeast extract medium; 2) malonate actually stimulated sporulation in MSG medium when added at a certain stage of growth.

The presence of an acetate condensing system essential to the growth initiation of cells of B. cereus var. mycoides grown in MSG medium is inferred by the studies with fluoroacetate. The condensation of two moles of glyoxylate via glyoxylate carboligase, shown to be functional in cells during early logarithmic growth, and subsequent metabolism by way of Kornberg's glycerate pathway (Kornberg, 1961) could provide a source of acetate as acetyl coenzyme A. Although direct evidence for an acetyl coenzyme A-glyoxylate condensation is lacking, the fact that malate can overcome the inhibition of growth by fluoroacetate suggests an active malate synthetase in which the competitive inhibition resulting from the formation of fluoromalate is relieved by malate. Although condensing enzyme was not studied, a malate synthetase reaction could preclude its requirement for growth and biosynthesis providing glutamate could be converted to glyoxylate without being shunted through the aconitase system of enzymes of the TCA cycle as suggested. The glyoxylate formed from glutamate may then proceed through the glycerate pathway to form acetyl coenzyme A which in turn could condense with glyoxylate via the malate synthetase reaction.

Ochoa (1948) and Barban and Ajl (1953) agree that extensive

biosynthesis of tricarboxylic acids can occur through the fixation of CO_2 into alpha-ketoglutarate. Although frequently referred to as a reductive carboxylation (Cantino and Horenstein, 1956; Wood and Stjernholm, 1962), the carboxylation of alpha-ketoglutarate, as pointed out by Ochoa (1948), may not truly be representative of such. The reaction considered here is a beta-carboxylation and should not be confused with what is normally thought of as a reductive carboxylation, which occurs by reversal of the oxidative decarboxylation of alpha-keto acids. Furthermore, the latter reaction type is associated with the generation of a high energy intermediate. The alpha-ketoglutarate carboxylation reaction proceeds in either direction in the absence of high energy intermediates.

The equilibrium of many isolated reactions occurring in metabolic systems may be far in one direction. However, if placed in their proper perspective as an integral part of a complex metabolic organization, the equilibrium of many such reactions can be shifted in the opposite direction providing: 1) a suitable energy source is available, and 2) the proper sequential reactions are functioning. Such is the case with the carboxylation of alpha-ketoglutarate to form isocitrate.

> While the equilibrium of this reaction is . . . in the direction of decarboxylation, it can be shifted in the opposite direction through reduction of oxalosuccinate to d-isocitrate by reduced triphosphopyridine nucleotide (TPNred) in the presence of isocitric dehydrogenase. If the oxidized TPN (TPNox) thereby formed is reduced by an independent dehydrogenase system of suitable oxidative-reduction potential, there occurs a further shifting of equilibrium in favor of CO_2 fixation. (Ochoa, 1948)

Ochoa used glucose-6-phosphate dehydrogenase in supplying NADPH to aid alpha-ketoglutarate carboxylation by pig heart. Since the reaction

requires a reduced pyridine nucleotide as a source of energy, B. cereus var. mycoides grown in MSG medium apparently utilizes glutamate dehydrogenase in an analogous manner.

Whether the reaction leading to the formation of alpha-ketoglutarate from isocitrate by way of intermediary oxalosuccinate is catalyzed by one or two enzymes remains unsettled. However, because of the instability of oxalosuccinate and since the activities of the two enzymes have not been separated, it is the general consensus that a single enzyme catalyzes both reactions. Thus, a one-step catalysis may occur where the oxidation of isocitrate results in a spontaneous decarboxylation without oxalosuccinate ever really appearing as an intermediate.

Two types of isocitrate dehydrogenases are known, one using NADP which is reversible and one using NAD which is irreversible. The reversible reaction would appear to be accomplished in two separate steps viz, carboxylation followed by reduction. For the express purpose of clarification, this reaction is discussed throughout as a two-step mechanism. Isocitrate dehydrogenase measured by the forward reaction showed highest activity in cells harvested just after germination, while the activity of alpha-ketoglutarate carboxylation in these cells was quite low. Two things could account for this: 1) the carboxylation of alpha-ketoglutarate is independent of isocitrate dehydrogenase, the reaction involving two distinct enzymes in which one is defunct because of some metabolic control mechanism such as feedback inhibition or repression, or 2) the forward reaction may be a single-step irreversible NAD dependent reaction which is totally independent of the reversible NADPH dependent

reaction.

Until now no important physiological function has been attached to the synthesis of isocitrate from alpha-ketoglutarate and CO_2 (Wood and Stjernholm, 1962). Coupled with the isocitrate lyase reaction and Kornberg's glycerate pathway, this reaction assumes greater importance by providing a system for energy and biosynthesis during growth in a medium containing glutamate as the sole carbon and nitrogen source.

The sharp declination of the rates of oxygen uptake observed with isocitrate and glyoxylate may denote the activity of more than one enzyme on these substrates with the inhibition of one reaction by a control mechanism. In the case of isocitrate, at least two of the enzymes could be isocitrate dehydrogenase and isocitrate lyase, both of which have been demonstrated in extracts of cells grown under similar conditions. Since the glyoxylate formed from the latter enzyme could condense with acetyl coenzyme A if malate synthetase is present, the acute deflection observed in the rate of oxygen uptake with isocitrate could be due to a diminished supply of endogenous acetyl coenzyme A. If this were so however, one would expect a more gradual effect. In the case of glyoxylate, Megraw (1964) demonstrated several different enzymes in B. cereus T acting on this substrate.

Powell and Strange (1956) detected an accumulation of alpha-ketoglutarate in cultures of B. cereus and B. subtilis before the onset of sporulation. The high endogenous rate of NADPH oxidation observed with extracts of B. cereus var. mycoides could be due to the carboxylation of residual alpha-ketoglutarate. Although dialyzed extracts were used,

which lessened endogenous NADPH oxidation, more extensive dialysis may have completely removed this effect, unless an NADPH oxidase is functioning, since the reaction sequence as described in Figure 7 is independent of exogenously supplied CO_2. The possibility of a direct reduction of alpha-ketoglutarate to form alpha-hydroxyglutarate cannot be completely eliminated except that no activity beyond the endogenous rate was noted in the absence of $NaHCO_3$.

The failure to actually demonstrate glutamate dehydrogenase activity in cells harvested during early logarithmic growth may be attributed to the coupling of the NADPH formed by this reaction with the reaction leading to the production of isocitrate. The low alpha-ketoglutarate dehydrogenase activity would augment this coupling. The increased alpha-ketoglutarate dehydrogenase activity in cells harvested during late logarithmic growth permits the detection of glutamate dehydrogenase because less alpha-ketoglutarate is available for carboxylation.

No cofactors were added to reaction mixtures containing undialyzed extracts. Many of the enzymes studied require different cofactors in minute quantities which undoubtedly would result in weighing and dilution errors of significant proportions. Although reaction rates might have been increased by the addition of cofactors, such additions would have also served to distort an already obscure picture of a very complex metabolic system.

Changes in levels of enzymatic activity of alpha-ketoglutarate dehydrogenase, isocitrate dehydrogenase, glutamate dehydrogenase, alpha-ketoglutarate carboxylation, glyoxylate carboligase, and succinate

dehydrogenase in cells harvested just prior to sporulation are evidence of shifting metabolic patterns. Studies of these particular enzymes have been neglected to a large extent by other investigators of sporulation.

As made evident by the literature, glutamate appears to be an important intermediate during sporogenesis in glucose media. One particularly interesting observation is that glutamate and glutamine stimulate the non-oxidative conversion of pyruvate to acetoin in B. subtilis (Keynan et al., 1954). The fact that acetoin formation precedes the formation of poly-beta-hydroxybutyrate just prior to sporogenesis (Kominek et al., 1963), coupled with the variations in glutamate concentration during growth and sporulation (Young and Fitz-James, 1959; Millet and Aubert, 1960), suggests that glutamate itself may play an important role in controlling reactions essential to spore formation. Complementing this is the early commitment to sporulation of cells grown in MSG medium.

It has not been conclusively established that sporulation requires energy. Since the TCA cycle is apparently functioning, at least in part, during sporulation its primary role in sporulating aerobic bacilli appears to be one of biosynthesis rather than energy-yielding. Metabolic patterns in aerobic and anaerobic spore formers may be more closely aligned than has been formerly realized. Growth in glucose by most species of Bacillus can certainly occur anaerobically provided that hydrogen acceptors are present or can be synthesized. Since the oxygen requirement for sporulation is generally considerably higher than that for growth (Roth et al., 1955) it could indeed be that growth occurs anaerobically among all spore formers, while sporulation in these same organisms demands increased

oxygen requirements. As yet, no conclusive evidence to the contrary has been reported.

SUMMARY

The strain of Bacillus cereus var. mycoides used in this investigation grew and sporulated well in a glutamate-salts medium (MSG medium). Kojic acid inhibited sporulation in this medium up to a point beyond which its effect was lessened.

Malonate showed no inhibitory effect on growth or sporulation in MSG medium but did inhibit sporulation in glucose and acetate media, indicating that the metabolic pathways leading to spore formation in the latter two media proceeded to a common point, that is, through the succinate dehydrogenase system. Malonate added during logarithmic growth stimulated sporulation in MSG medium. Some of the TCA cycle enzymes were repressed to some degree in cells grown in MSG-malonate medium.

Fluoroacetate inhibited growth and sporulation when added during the lag or logarithmic phase of growth suggesting an acetate-condensing system essential to early vegetative growth in MSG medium. The diminishing inhibitory effect of later additions of fluoroacetate could have been due to shifts in metabolic pathways, the excretion of a compound or compounds into the medium which overcame the inhibition, or a combination of both. Reinoculated logarithmic culture filtrates of Bacillus cereus var. mycoides grown in MSG medium showed no inhibition by fluoroacetate which may have been due to the excretion of poly-beta-hydroxybutyrate, the concentration of which has been shown by other investigators to increase during logarithmic growth and to decrease just prior to visible sporulation.

The addition of bicarbonate to a slightly alkaline MSG medium stimulated the sporulation of Bacillus cereus var. mycoides.

Cells harvested during early logarithmic growth metabolized glutamate to isocitrate via a partial reversal of the TCA cycle which involved the carboxylation of the alpha-ketoglutarate formed from an active glutamate dehydrogenase. The product of this reaction is coupled with the NADPH generated by the glutamate dehydrogenase which accounts for the failure to actually demonstrate glutamate dehydrogenase in cells harvested during early logarithmic growth. The cells at this stage of growth revealed low alpha-ketoglutarate dehydrogenase and succinate dehydrogenase activity when compared with cells harvested during late logarithmic growth. These results, along with those obtained with malonate, suggested a rather unique route for glutamate utilization which bypassed the succinate dehydrogenase system.

Isocitrate lyase was shown to be active in cells during early logarithmic growth in MSG medium, while cells harvested from glucose medium during a comparable stage of growth revealed no activity of this enzyme. Thus, the inhibition observed on sporulation when malonate was incorporated into the glucose and acetate media, and the lack of inhibition when it was added to MSG medium, is explained by the different pathways the organism takes during growth in the different media. Growth in MSG by the organism involves a pathway yielding succinate from isocitrate via isocitrate lyase. Thus, if enough succinate is formed during growth, the inhibition imposed by malonate on succinate dehydrogenase is overcome during sporogenesis. Growth of the organism in glucose medium, and probably acetate medium as well, occurred without the formation of succinate. Hence, the succinate dehydrogenase reaction was blocked

when it may have been critically required for the formation of spore material.

Glyoxylate carboligase, active in cells harvested from MSG medium during early logarithmic growth suggested that subsequent metabolism proceeded through Kornberg's "glycerate pathway". The "glycerate pathway" was also indicated to be functioning in Bacillus cereus T when grown in a glutamate medium (Megraw, 1964).

Cells undergoing initial outgrowth in MSG medium showed the greatest activity of isocitrate dehydrogenase while the activity of alpha-ketoglutarate carboxylation in these cells was quite low. Two things could account for this: 1) the carboxylation of alpha-ketoglutarate is independent of the isocitrate dehydrogenase reaction, the reactions involving two distinct enzymes, one being defunct because of some metabolic control mechanism, or 2) the forward reaction may be a single-step irreversible NAD dependent reaction which is totally independent of the reversible NADPH dependent reaction leading to isocitrate formation.

The organism grown in MSG medium was shown to be committed to sporulation soon after the initiation of growth by virtue of the ability of the washed cells to sporulate in the presence of glucose and distilled water.

It was speculated that perhaps the metabolic relationship of aerobic spore formers and anaerobic spore formers is more closely related than is realized.

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